AN INVESTIGATION INTO SOME ASPECTS RELATED TO THE PHYTOREMEDIATION POTENTIAL OF *Pinus Radiata* (D. Don) & *Chamaecytisus proliferus* (L.f.) Link ssp. *proliferus* var. *palmensis* (H. Christ.)

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ABSTRACT

Both *Pinus radiata* and *Chamaecytisus palmensis* were inducted into tissue culture. *In vitro* clonal shoots were generated from cotyledon-derived meristematic cultures of *P. radiata.* Attempts were made to induce shoot organogenesis in *C. palmensis* explants, on solid media supplemented with a range of plant growth regulators. *In vitro* clonal shoots of *C. palmensis* were generated from seedling explants.

The potential for Pb phytoextraction was investigated in seedlings and clones of *P. radiata* and *C. palmensis*. Aspects of tolerance, uptake, and translocation of Pb were studied within each species and in comparison to each other. Effects of Pb on different aspects of plant growth were examined including inhibition of seed germination, root growth inhibition, fresh weight changes of *in vitro* tissue, and responses of seedlings and excised shoots in solution culture. Both *P. radiata* and *C. palmensis* were insensitive to Pb during seed germination. Pb inhibited formation of lateral roots and inhibited root and shoot elongation in *P. radiata* seedlings. *C. palmensis* displayed greater tolerance to Pb than *P. radiata* during early seedling growth.

Plants growing in hydroponic culture were exposed to $Pb(NO_3)_2$, with and without the addition of the chelating agents H-EDTA and EDTA. Subsequently treated materials were examined for Pb uptake by flame atomic absorption spectrometry. Both *P. radiata* and *C. palmensis* accumulated unchelated Pb predominantly in root tissue, while Pb, in the presence of H-EDTA or EDTA, was taken up principally by the shoots in both species.

With transmission electron microscopy, ultrastructural observations were carried out on ultra-thin sections, derived from tissues treated with Pb in the presence and absence of chelators, from both *P. radiata* and *C. palmensis*. In *P. radiata,* Pb was deposited in cell walls in root tissues; and in intercellular spaces in shoot tissues. In *C. palmensis*, Pb was found in cell walls, bacteroids and mitochondria in root nodule tissue; in middle lamellae and intercellular spaces in root tissues; and in chloroplasts, pit membranes, and plasmodesmata in shoot tissues.

When acid phosphatase activity, in response to Pb exposure, was examined in both species, no substantial differences were found with regard to treatment but some evidence was found of differences in response among *P. radiata* clones. No new isozymes of acid phosphatase were found in *C. palmensis* in response to Pb but evidence was found that root isozyme activity increased in response to unchelated Pb.

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LIST OF ABBREVIATIONS

2,4-D	2,4-dichloro-phenoxy-acetic acid
APS	ammonium persulphate
BA	benzyladenine
Bis	N,N'-methylene bisacrylamide
BSA	bovine serum albumin
dH_2O	distilled water
DMF	dimethylformamide
DW	dry weight
EDTA	ethylenediaminetetraacetic acid disodium salt
EPXMA	electron probe x-ray microanalysis
FRI	Forest Research Institute, N.Z.
FW	fresh weight
HC	Huang and Cunningham nutrient solution
H-EDTA	N-(2-hydroxyethyl) ethylenediamine triacetic acid
IBA	indol-3-ylbutyric acid
LP	modified Quoirin and LePoivre nutrient medium
LP5	LP plus 5 mg L^{-1} BA
MS	Murashige and Skoog nutrient medium
NAA	α-naphthalene acetic acid
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
ppm	parts per million
PPM	Plant Preservative Mixture
PVPP	polyvinyl polypyrrolidone
REM	root elongation medium
RIM	root induction medium
SEM	scanning electron microscope
TCE	trichloroethylene
TDZ	1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea
TEM	transmission electron microscope
TEMED	N,N,N',N'-tetramethylethylenediamine
TRIS	Tris-HCl
V/V	volume for volume
XP	(5-bromo, 4-chloro, 3-indolyl-phosphate-ρ-toluidine)

CHAPTER I

INTRODUCTION

Since the dawn of humanity people have utilised natural resources at an ever increasing rate and concomitant with the increase in population has been a rise in the level of waste products from human activities, in essence, pollution.

From the advent of the industrial revolution, a little over 200 years ago, pollution of the biosphere with toxic substances has accelerated dramatically, and foremost amongst those substances are toxic heavy metals.

(1) HEAVY METALS

In the last 100 years human activities such as mining, burning of fossil fuels, dumping of municipal wastes, manufacture and application of fertilisers and pesticides, and sewage disposal have seen the release of ever increasing amounts of toxic heavy metals into the environment (Freedman and Hutchinson, 1981; Salt *et al.*, 1995; Nriagu, 1979; Raskin *et al.*, 1994).

The term 'heavy metal' is widely used but means different things to different scientists (Ormrod, 1984). It originates from a categorisation by density which is hardly biologically significant. A perhaps more useful and less emotive term is 'trace metal', which generally implies a metal found in low concentration, perhaps ppm. or less, in some specified source, e.g. soil, plant, tissue, ground water, etc. (Phipps, 1981). Where possible the term trace metal is used in preference to either heavy metal or toxic metal.

The natural concentration ranges of most trace metals in soils are wide, the main sources are the parent materials from which the soils are derived. These are usually weathered bedrock or overburden transported by wind, water or glaciation, which may be of local or exotic origin. At times anthropogenic inputs may exceed those from natural geological sources (Nriagu, 1979; Thornton, 1981).

1.1 Trace metals and plants

The list of elements essential for plant nutrition (one without which plants cannot complete their life cycle) is still a subject of some controversy (Raskin *et al.*, 1994) but about 12 elements present in rocks and soils, normally in very small amounts, are essential for plant nutrition: P, K, S, B, Cu, Fe, Mn, Mo, Si, V, Zn and possibly Ni are required for plants (Thornton, 1981).

The elemental composition of plants is very different from that of the soil in which they grow. Most of these differences can be attributed to a plant's ability to fix carbon from the air and to absorb essential macro- and micro-nutrients from the soil. In many ways, living plants can be compared to solar-driven pumps that can extract and concentrate certain elements from their environment (Raskin *et al.*, 1994). In addition to the uptake of those elements that are essential for plant nutrition, many non-essential trace metals are found in field-grown plants. These include Cd, Hg, Cr, Co and Pb (Raskin *et al.*, 1994). However, foliar uptake of airborne trace metals has been demonstrated in several species (Ormrod, 1984; Ernst, 1976).

1.2 Occurrence and uses of lead

In normal soil in Britain, the range of Pb concentration is 10-150 ppm. whereas in mineralised, metal-rich soil the concentration may be as high as 1000 ppm., or 1% (Thornton, 1981). Contamination by industrial Pb appears to have occurred everywhere on earth. The main means of dispersal of this Pb are atmospheric transport of aerosols from smelters and petrol exhausts, and re-entrained dusts and smokes. Studies have found clearly defined gradients of Pb contamination beside roadways (Daines *et al.*, 1970). This source of Pb contamination has principally been caused by the use of leaded petrol (Atkins, 1969). Pb is currently used in storage batteries, plumbing installations, for containing corrosive liquids, for electrical cable coverings, ammunition manufacture, paints and glazes, mining and smelting, glass manufacture, for

sealing wine corks and, as Pb is relatively impenetrable to high-energy radiation, protective shields for industrial workers and researchers handling radioactive materials, e.g. radiologists, biochemists etc. (Chang, 1991).

1.3 Lead and human health

Pb has no known beneficial function in human metabolism. Pb is extremely toxic, its effect on humans is cumulative. Pb is absorbed slowly and incompletely from the gastrointestinal tract, and can also be absorbed from the respiratory tract when inhaled. It is known that Pb ions inhibit enzymes that catalyse the reactions for the biosynthesis of haemoglobin, consequently one symptom of Pb poisoning is anaemia. Brain damage is the most common symptom of those, especially children, suffering from acute Pb poisoning. (Chang, 1991).

(2) LAND CONTAMINATION

For many years, there was a general lack of concern for the environment and a widespread but unfounded assumption that the subsurface environment would adsorb or degrade almost unlimited amounts of chemical contaminants. In the past, prevailing popular views held that the passage of water through soil exerted a purifying effect and that wastes dumped into the ground somehow were cleansed from the system (Suthersan, 1997). The main classes of contaminants that can be released to the subsurface and cause an adverse impact include organic compounds, inorganic compounds, and elements, including metals.

Metals occur as natural constituents in soils but metals may also enter the soil via beneficial agricultural additives such as lime, fertiliser, manure, herbicides, fungicides, and irrigation waters. Metal-containing waste materials that impact soil and groundwater pollution include sewage sludge, storm water run-off, dredged materials, wastes from mining and smelting operations (Oyedele

et al., 1995), filter residues from wastewater treatment and atmospheric emission control, ashes and slags from burning of coal and oil, and hazardous wastes from municipal refuse. Some of the primary industrial activities that cause metal contamination are metal plating, tanning, and timber treatment (Bolan and Thiagarajan, 1998). The common metals causing environmental concern when present in the subsurface environment are Cr, Cd, Zn, Pb, Hg, Ni, Cu, and Ag (Suthersan, 1997).

2.1 Land remediation strategies

In general terms, the options available are to remove, render harmless or contain the sources of contamination. Remediation options can be classified in a number of ways but in the first instance the question is whether the material is to be excavated or left *in situ*. The processes available for treatment of soils are, in principle, the same whether carried out *in situ* or after excavation. Broadly, the options are:

(1) Excavation for deposition elsewhere

(2) Soil treatment methods (*ex situ*)

(3) *In situ* treatment methods

(4) Isolation or macro-encapsulation

2.1.1 Excavation

Excavation of the contaminated material and its replacement with clean fill may be the most satisfactory solution when the contamination is fairly shallow and the volume relatively small. However, complications may arise that preclude this option e.g., the contaminant may have moved out of the site and possibly beneath neighbouring buildings, there may be no sharply defined boundary to the contaminated zone, or there may be difficulty finding a disposal site or suitable clean fill material.

2.1.2 *Ex situ* and *in situ* soil treatment

In general, potential soil treatments, both *ex situ* and *in situ*, fall into one of several categories (Smith, 1987; Suthersan, 1997):

(a) Physical: Solvents, gravity, mechanical, vacuum, precipitation.

(b) Thermal: Desorption, steam stripping, heating, incineration.

(c) Chemical: Hydrolysis, neutralisation, oxidation/reduction.

(d) Stabilisation/Solidification: Cement, microencapsulation, capping.

(e) Biological: Bioremediation, phytoremediation.

Many of these techniques apply mainly to organic contaminants, both volatile and semi-volatile, and are not particularly well suited to the removal of trace metals.

2.1.3 Macroencapsulation/isolation

Macro-encapsulation simply means putting a container around the contamination. This may surround the whole site- top, sides and bottom, but frequently only partial encapsulation or containment is carried out (Smith, 1987). Often it is only possible to place a cover or 'cap' over the contaminated zone, as in the case of abandoned dumps. Modern landfills are almost always constructed with a bottom liner system that includes a leachate collection layer. Leachate is the contaminated liquid that drains from the waste material (Koerner and Daniel, 1997).

2.2 Remediation costs

The costs associated with soil remediation are highly variable and depend on the contaminant, site conditions and the volume of material to be remediated. Metal contaminants are notoriously hard to remediate. Techniques that remediate a soil *in situ* are generally less expensive than those that require excavation. On average, remediation costs are US$10-100 per m^3 of soil for volatile or water-soluble pollutants remediated *in situ*, US$60-300 per m^3 for compounds handled by landfilling or low-temperature thermal treatment, and US$200-270 per m^3 for

materials requiring special landfill arrangements or high temperature thermal treatments. Certain materials, e.g. radionuclides, require even more intensive management techniques that can cost well beyond US$1000-3000 per m^3 of soil (Cunningham *et al.*, 1995).

(3) BIOLOGICAL APPROACHES

3.1 Microorganisms

Bioremediation involves the application of microorganisms in the clean up of hazardous substances present in the environment. Biological processes, which involve enzymes as catalysts, occur naturally, and can modify organic contaminant molecules such as aliphatic hydrocarbons, aromatic hydrocarbons and chlorinated hydrocarbons. The major agents causing the biological transformations in soil, sediment, surface water and groundwater, both under aerobic and anaerobic conditions, are the indigenous microorganisms that inhabit these environments. Communities of bacteria and fungi can degrade a multitude of synthetic compounds and probably every natural product (Suthersan, 1997). *In situ* bioremediation works by turning the contaminated site into a biofermentor. Introduced microorganisms, or those already on the site, detoxify the pollutant either by using it as the sole source of carbon or energy or both, or more usually, by degrading the contaminant as a co-substrate for growth. Bioremediation often requires significant engineering costs to produce an environment in which microorganisms can grow. Exogenous carbon and energy sources are frequently needed, and engineering of the appropriate oxidation-reduction potential, temperature, and water potential are required for fermentation to occur (Stomp *et al.*, 1993). However, problems have been encountered in attempts to apply microbial biotechnology to metal remediation and recovery. For example, whereas biological and chemical processes can possibly transform toxic organic substrates into innocuous end products, such as CO_2, metals may be changed only in valence or chelation state by these agents and they still remain as the same

metal. Potentially bioremediation may lead to fixation of the metal, i.e. conversion to a very stable insoluble form (e.g. a sulphide), so that mobility of the metal in the biosphere is severely diminished (Summers, 1992). Ultimately though, microorganisms alone do not have the ability to translocate metals out of the zone of contamination.

3.2 Microorganisms and plants

In unmanaged natural ecosystems, soil microorganisms are often found in symbiotic relationships with plants. Root surfaces support active bacterial biofilms and fungal proliferations generally referred to as the rhizosphere. The rhizosphere functions as a symbiotic system in which plant roots supply organic nutrients and energy through root exudates, and acquire inorganic nutrients such as phosphate. Plant roots also have a major impact on soil oxidation-reduction potential, either directly by transporting oxygen via roots, or indirectly by changing soil porosity over time, thus providing an oxidation-reduction environment suitable for microorganisms. Plants modulate swings in soil water potential through transpiration, by the continual addition of water-retentive organic matter, and they provide soil cover which dampens temperature fluctuations. In return, the rhizosphere microorganisms greatly enlarge the root's effective surface area and uploading capacity. They also partially regulate root metabolic capacities and can alter most measurable soil physical and chemical parameters.

Rhizosphere microorganisms can detoxify pollutants directly, with growth supported by root exudates. The increased microbial activity in the rhizosphere has been shown to translate into greater metabolism of organic contaminants, e.g., trichloroethylene (TCE), in vegetated vs. non-vegetated soils (Aprill and Simms, 1990; Walton and Anderson, 1990). As well as improving remediation rates by enhancing rhizosphere symbiosis, plants can potentially function directly in detoxification. Through transpiration, water moves into the roots following a negative pressure gradient, rises through the vascular tissue of the stem and

branches and is finally evaporated from the leaf surface. Contaminants in the soil water can potentially be taken up by the roots and translocated within the plant (Stomp *et al.*, 1993).

3.3 Plants

The notion that plants can tolerate toxic materials in the soil in which they grow is well established (Cataldo and Wildung, 1978). Natural mineral deposits containing relatively large quantities of trace metals are present in many regions of the globe. These areas often support very characteristic plant assemblages and species that thrive in these metal-enriched environments (Bradshaw, 1952). Examples of such distinct plant communities include serpentine, seleniferous, uraniferous and calamine floras (Raskin *et al.*, 1994). As a result of their association with specific ore deposits, many metallophyte plants have been used as so-called indicator species in prospecting for mineral deposits (Peterson, 1971; Brooks, 1983).

Three basic strategies are employed by plants growing on metalliferous soils (Baker and Walker, 1990). Metal **excluders** effectively prevent metal from entering their aerial parts over a broad range of metal concentrations in the soil, however they can still contain large amounts of metal in their roots. Metal **non-excluders** actively accumulate metals in their above ground tissues and can be roughly divided into two groups: **indicators** and **hyperaccumulators** (Baker, 1981). Metal levels in the tissues of indicator species generally reflect metal levels in the soil in which they grow.

3.3.1 Hyperaccumulators

Hyperaccumulators can concentrate metals in their above-ground tissues to levels far exceeding those present in the soil or in non-accumulating species growing nearby. One definition proposes that a plant containing more than 0.1% of Ni, Co, Cu, Cr or Pb, or 1% of Zn or Mn, in its leaves on a dry weight basis should be designated a hyperaccumulator, irrespective of the metal concentration

in the soil (Baker and Brooks, 1989). Little is known of the biological and evolutionary significance of metal hyperaccumulation but several theories have been advanced including: inadvertent uptake, drought resistance, tolerance or disposal of metal from plants, and most strongly favoured, as a defence mechanism against herbivores or pathogens (Baker *et al.*, 1988).

The first hyperaccumulators characterised were members of the Brassicaceae and the Fabaceae and in recent years the list of hyperaccumulating plants has expanded greatly with Ni accumulators being the most abundant followed by Co, Cu and Zn. One of the most striking examples of metal hyperaccumulation is the New Caledonian tree *Sebertia acuminata* which accumulates 25% Ni in its latex on a dry weight basis (Jaffre *et al.*, 1976) but most examples are less spectacular. For example, *Thlaspi caerulescens*, a member of the Brassicaceae, can accumulate up to 4% Zn in its tissue without apparent damage (Brown *et al.*, 1994) and putative Pb accumulators such as *Thlaspi rotundifolium* or *Brassica juncea* have been reported to accumulate about 0.8-1.0 % Pb dry weight (Reeves and Brooks, 1983). Currently at least 45 plant families are known to contain metal-accumulating species and the number of metal-accumulating taxa identified to date has grown to at least 397 (Salt *et al.*, 1998).

(4) METAL RESISTANCE MECHANISMS

4.1 Natural chelation

A large proportion of metals in soils are relatively unavailable to plant roots as they are bound to organic soil constituents, inorganic soil constituents, or they are present as insoluble precipitates. To accumulate these 'soil-bound' metals plants must first mobilise them into the soil solution which can be achieved in a number of different ways. Metal chelating molecules known as phytosiderophores can be secreted into the rhizosphere to chelate and solubilise 'soil-bound' metals. Mugineic acid and avenic acid serve as phytosiderophores of plants from the Poaceae family (Kinnersley, 1993). These substances are released

in response to Fe and Zn deficiency and can mobilise Cu, Zn, and Mn from soil (Romheld, 1991). Dicotyledonous and monocotyledonous species, excluding grasses, deal with Fe deficiency with the so called 'strategy I' system which is characterised by three components: a plasma membrane-bound inducible reductase, enhanced net excretion of protons, and in many instances enhanced release of reductants or chelators, which are mainly phenolics (Marschner and Romheld, 1994). The phytotoxicity of metal ions can be reduced with chelation by specific high-affinity ligands reducing the solution concentration of free metal ions. Two major classes of metal chelating peptides are known to exist in plants - **metallothioneins** and **phytochelatins**. Metallothioneins are gene-encoded, low molecular weight, cysteine-rich polypeptides (Robinson *et al.*, 1993). Phytochelatins are low molecular weight, enzymatically synthesised cysteine-rich peptides known to bind Cd and Cu in plants (Grill *et al.*, 1985; Grill *et al.*, 1987; Jackson *et al.*, 1987; Steffens, 1990; Rauser, 1995; Maitani *et al.*, 1996). Although not strictly defined as chelation, precipitation of Zn as Zn-phytate has also been suggested as a Zn detoxification mechanism and it is also likely that intra- and extracellular precipitation of Pb as carbonates, sulphates, and phosphates plays a role in the detoxification of this metal in plant tissues (Salt *et al.*, 1998).

4.2 Root uptake and transport

Solubilised metal ions may enter the root either via the extracellular (apoplastic) or intracellular (symplastic) pathways. Apoplastic transport is limited by the high cation exchange capacity of cell walls, unless the metal ion is transported as a non-cationic metal chelate, such as Cd-citrate (Senden *et al.*, 1990). Symplastic transport requires that metal ions move across the plasma membrane. Most metal ions enter plant cells by an energy-dependent, saturable process via specific or generic metal ion carriers or channels (Clarkson and Luttge, 1989). Non-essential trace metals may effectively compete for the same transmembrane carriers used by essential metals. This relative lack of sensitivity

in trans-membrane ion transport may partially explain why non-essential trace metals can enter cells, even against a concentration gradient.

Once metal ions have entered the root they can either be stored or exported to the shoot. Metal transport to the shoot primarily takes place in the xylem but there is some evidence that redistribution may occur in the phloem (Stephan and Scholz, 1993). To enter the xylem, metal ions must first cross the Casparian strip, a layer of suberin-impregnated endodermal cells which divides the cortex and the vascular cylinder. To cross this strip of water-impermeable cell wall, metal ions must move symplastically within the endodermal cells, as apoplastic transport is blocked (Raven *et al.*, 1986). The possibility exists, therefore, that symplastic transport of metals within the endodermis is a rate limiting step in metal translocation to the shoot. Recent evidence from work with Ni hyperaccumulators from the genus *Alyssum* suggests that xylem loading of Ni may be facilitated by the binding of Ni to free histidine (Kramer *et al.*, 1996). The involvement of organic acids in metal transport in certain metal accumulators has been demonstrated by analysis of xylem sap (Baker and Brooks, 1989). The non-proteinaceous amino acid nicotianamine is ubiquitous among plants and has the ability to form complexes with various divalent metal ions including Cu, Ni, Co, Zn, Fe, and Mn (Stephan and Scholz, 1993; Stephan *et al.*, 1996). Movement of metal ions, particularly Cd, in xylem vessels appears to be mainly dependent on transpiration-driven mass flow (Salt *et al.*, 1995a).

4.3 Compartmentalisation

Once trace metals accumulate within cells they need to be detoxified and one of the ways this can occur is through compartmentalisation e.g., Zn may be chelated to organic acids and accumulated within the vacuole (Mathys, 1977) and intact vacuoles isolated from tobacco exposed to Zn were shown to contain high levels of Zn (Krotz *et al.*, 1989). This has also been confirmed in roots and shoots of the Zn accumulator *Thlaspi caerulescens* (Vasquez *et al.*, 1994). Zn exposure can lead to an increase in the vacuolar volume fraction of meristematic cells

within the root tip of *Festuca rubra* (Davies *et al.*, 1991). Cd is known to accumulate in vacuoles (Vogeli-Lange and Wagner, 1990), where it associates with phytochelatins (Steffens, 1990), and this accumulation appears to be driven by a Cd/H antiport and an ATP-dependent phytochelatin transporter (Salt and Wagner, 1993; Salt and Rauser, 1995b). Leaf trichomes also appear to provide a site for the sequestration of Cd (Salt *et al.*, 1995a), Mn (Blamey *et al.*, 1986), and Pb (Martell, 1974).

Ultrastructural studies have shown that Pb taken up by plant roots tends to accumulate in the cell wall in *Allium cepa* (Antosiewicz and Weirzbicka, 1999; Wierzbicka, 1998) and *Anthoxanthum odoratum* (Qureshi *et al.*, 1986). This ability of the cell wall to bind Pb is thought to play some role in Pb tolerance and this was confirmed by removal of the cell wall and exposure of the isolated protoplasts to Pb (Poulter *et al*, 1985). Pb also accumulates in vacuoles in *Allium* sp. (Antosiewicz and Weirzbicka, 1999) and in tissue cultured cells of *Populus* sp. (Ksiazek *et al.*, 1984).

(5) THE RISE OF PHYTOREMEDIATION

For a long time, the ability of plants to accumulate metals was considered a detrimental trait. As metal-accumulating plants are at the bottom of many natural food chains they are directly or indirectly responsible for a significant proportion of the dietary uptake of toxic trace metals by humans and other animals (Kabata-Pendias and Pendias, 1989). So, initially the concept of metal hyperaccumulation by plants was regarded as an interesting botanical curiosity with little application beyond the plant's ability to either 'indicate' the presence of ore bearing minerals (Antonovics *et al.*, 1971; Brooks, 1972; Baker, 1981) or potentially serve as monitors of metal pollution (Cataldo and Wildung, 1978). This interest evolved into the idea that metal-tolerant plants could be used to reclaim toxic metalliferous waste land by vegetative cover (Smith and Bradshaw, 1979). By the early 1980s scientists were beginning to formalise concepts of permanent soil remediation through plant extraction of metals such as Cd

(Utsunomiya, 1980) and it was the existence of the hyperaccumulation phenomenon that inspired the concept of phytoextraction (Chaney, 1983). By 1991 the term **phytoremediation** had been coined to describe the emerging set of technologies that encompass the use of green plants to remove, contain, or render harmless environmental contaminants. The concept itself is not new, constructed wetlands, reed beds and floating plant systems have been common for the treatment of some types of wastewater for many years. Phytoremediation can be applied to both organic and inorganic pollutants, present in solid substrates (e.g. soil), liquid substrates (e.g. water), and the air. Currently phytoremediation is divided into the following areas (Salt *et al.*, 1998):

- **Phytoextraction:** the use of pollutant-accumulating plants to remove metals or organics from soil by concentrating them in the harvestable parts.
- **Phytodegradation:** the use of plants and associated microorganisms to degrade organic pollutants.
- **Rhizofiltration:** the use of plant roots to absorb and adsorb pollutants, mainly metals, from water and aqueous waste streams.
- **Phytostabilisation:** the use of plants to reduce the bioavailability of pollutants in the environment.
- **Phytovolatilisation:** the use of plants to volatilise pollutants.

5.1 Advantages of phytoremediation

First and foremost is cost. Growing plants is envisaged as costing 2-4 orders of magnitude less than conventional engineering strategies of excavation and burial (Cunningham and Ow, 1996). It is well suited to treat large expanses of moderately contaminated soil where excavation is not possible, with minimal environmental disruption (Negri and Hinchman, 1996). It has applicability to a range of toxic metals, organics and radionuclides, eliminates secondary air or water-borne wastes and is publicly acceptable (Raskin *et al.*, 1994). As a rule, plants will survive higher concentrations of hazardous wastes than will most microorganisms used for bioremediation (Schnoor *et al.*, 1995). The accumulation

of significant amounts of metals in plant tissues has been referred to as 'biomining' and has the potential to provide not only solutions to remediation problems but also new dimensions in the mining of certain metals. The 'bio-ore' product contains both metal and the fuel for its own smelting (Cunningham and Berti, 1993).

5.2 Limitations of phytoremediation

Phytoremediation has a number of inherent technical limitations, e.g. the contaminant must be within, or be drawn toward, the root zones of plants that are actively growing (Negri and Hinchman, 1996). This restricts the method to the depths reached by the roots of the chosen species and is most suited for sites with shallow contamination, i.e. less than 5 metres depth (Schnoor *et al.*, 1995), although it has long been recognised that phreatophytic plants (plants known for fast growth and high water usage rates) such as willow (*Salix* sp.), alfalfa (*Medicago sativa),* and cottonwood (*Populus deltoides*) are effective at rooting deeply, to 30 metres plus (Nyer and Gatliff, 1996). Contaminants that are highly water soluble may leach outside the root zone and require containment (Cunningham *et al.*, 1995). The amount of contaminant removed is dependent on the ratio of metal concentration in the harvested parts to total harvested biomass. Naturally occurring hyperaccumulators have slow growth rates, relatively low biomass and lack the ability to accumulate the most environmentally important metallic pollutants such as Pb and Cd (Salt *et al.*, 1998).

Phytoremediation is generally slower than physico-chemical methods and may need to be considered as a long term process, perhaps with sequential cropping over a number of seasons. When contaminants accumulate in leaves there is potentially a problem with leaf drop, particularly with deciduous species. Abscised conifer needles, however, are not thought to be readily dispersable by wind (Entry *et al.*, 1993). Although translocation of contaminant to the above-ground parts is the most desirable situation, roots may accumulate more contaminant than shoots and this may make harvesting more difficult

(Cunningham *et al.*, 1995). There is a need to prevent consumption of remediator plants by animals during remediation operations and any exotic plants introduced for phytoremediation must be prevented from becoming weeds. The biomass that is generated must be reduced in volume and disposed of in an ecologically sound manner.

5.3 Phytoextraction of trace metals

The optimum plant for the phytoextraction process should not only be able to tolerate and accumulate high levels of trace metals in its harvestable parts but also have a rapid growth rate and the potential to produce a high biomass in the field. For the technology to be viable within reasonable time frames, desirable rates of metal accumulation are required to be in the 1-2% (dry weight) range (Cunningham *et al.*, 1995). In recent years various research groups have investigated the responses of a number of different plant species, from widely diverse taxonomic groups, to a range of soil contaminants. Efforts have focused initially on the comparison of responses of known hyperaccumulators, such as *Thlaspi caerulescens* and *Brassica juncea*, with non-hyperaccumulators, in the uptake of metals like Zn (Baker *et al.*, 1994), Zn and Cd (Brown *et al.*, 1994; Brown *et al.*, 1995a; Ebbs *et al.*, 1997). Other approaches have included the use of trees such as *Pinus ponderosa* and *Pinus radiata* in studies on accumulation of ^{137}Cs and ^{90}Sr (Entry *et al.*, 1993).

Currently there are two basic strategies of phytoextraction being developed: chelate-assisted phytoextraction (induced phytoextraction), and long-term continuous phytoextraction. Field trials have been conducted using both strategies but chelate-assisted phytoextraction is more developed and is currently being implemented commercially, particularly for Pb. Estimates suggest that plants can remove between 180 and 530 kg ha^{-1} of Pb per year (Huang and Cunningham, 1996; Blaylock *et al.*, 1997), making remediation of sites contaminated with up to 2500 mg Pb g^{-1} soil possible in under 10 years.

(6) PLANTS AND LEAD

There is a widely held belief among research workers that Pb is very toxic to plants yet the results of laboratory studies conflict with field observations. There are actually no symptoms of Pb poisoning observed in plants growing under natural conditions, even when such plants contain considerable amounts (some hundred ppm) of Pb in their tissues (Koeppe, 1981).

6.1 Whole plant effects

Some of the early work on whole plant effects included responses of barley to Pb, (Keaton, 1937), assimilation of spray residues on crops (Jones and Hatch, 1945), characterising naturally tolerant species (Bradshaw, 1952) and development of techniques to measure Pb tolerance (Wilkins, 1957). In the 1960s some researchers looked at effects caused by Pb originating from car exhausts (Cannon and Bowles, 1962) while others were interested in effects on food crops (Warren and Delavault, 1962). Schuck *et al.* (1970) examined several crop species grown in close proximity to a busy highway and concluded that little translocation from root to shoot occurred. Miller *et al.* (1971), determined Pb uptake in corn, but also examined isolated organelles (mitochondria), and interactions with phosphate. This study demonstrated that plants do have the ability to take up, translocate and accumulate significant amounts of Pb in leaves.

In addition, the relationship between phosphate levels and Pb precipitation/availability was studied. The conclusion reached here and in other related research (Koeppe and Miller, 1970) was that physiological effects due to Pb, and uptake of Pb, in corn, varied inversely with the phosphate concentration of the nutrient solution, and with the phosphate status of the plant itself. Other groups looked at Pb uptake by crops in relation to other nutrients, such as lime, added to a Pb-contaminated soil (Cox and Rains, 1972), and both lime and N in combination with Pb (John and Van Laerhoven, 1972). Raising the pH of the soil by liming was found to decrease Pb availability and thus plant uptake. Rather than

quantify Pb uptake, some groups examined aspects of plant responses to Pb exposure e.g., soybean metabolism, as measured by pod fresh weight, was found to be inhibited by Pb and Cd, as were photosynthesis, protein and carbohydrate content (Huang *et al.*, 1974). Mukherji *et al.* investigated the effect of Pb on rice seedling growth. They noted inhibition of elongation and germination (Mukherji and Maitra, 1977). A comprehensive study was conducted on 25 species from 12 families to determine the extent to which seed coats are a barrier to Pb, and to what degree germination is affected by this metal (Wierzbicka and Obidzinska, 1998a). The major conclusion was that seed coats are selectively permeable to Pb ions.

Other examples in which general decreases in growth parameters have been determined after Pb treatment in hydroponic culture include studies with red maple and loblolly pine (Davis and Barnes, 1973), oats and rice (Fiusello and Molinari, 1973), corn and sunflower (Carlson *et al.*, 1975), and groundsel (Briggs, 1976). In a study on barley and maize seedlings, investigating concentration-dependent responses to $Pb(NO_3)_2$ in solution culture (Sobotik *et al.*, 1998), no effect was seen until the concentration was raised to 10^{-3} M, which partially inhibited roots but did not affect shoots. At 10^{-2} M, root growth was severely inhibited and shoot growth was affected. Almost no Pb penetrated the endodermis.

6.2 Specific plant effects

Many types of studies have been undertaken where plants were exposed to high concentrations of Pb in the rooting medium, and processes other than general growth were measured. These include reports on gas exchange and photosynthesis in sunflower (Bazzaz *et al.*, 1974), and corn (Bazzaz *et al.*, 1975), where reduced rates of photosynthesis were found with increased soil Pb concentrations. $PbCl_2$-induced pigment concentration changes were examined in red maple (Davis and Barnes, 1973) where anthocyanin content increased as much as 270% and in another quantitative study, $Pb(NO_3)_2$ in solution culture for 21 days caused a

decrease in chlorophyll content of oats of approximately 26% (Fiusello and Molinari, 1973).

6.2.1 Enzymes

Pb (6×10^{-2} M) in the liquid media of germinating rice decreased activity of protease, α-amylase and RNase but stimulated activity of DNase (Mukherji and Maitra, 1976). In a study of enzymes in soybean leaves, Lee *et al.* (1976) found that $Pb(NO_3)_2$ at 100 μg ml^{-1} for 10 days stimulated acid phosphatase, peroxidase, malic dehydrogenase and α-amylase but depressed levels of glutamine synthetase. Similarly, Pb-treated young corn plants exhibited increased acid phosphatase activity in the leaves and decreased activity in the roots (Maier, 1978).

In a study on the effect of Pb on nitrate reductase activity in mungbeans (*Vigna radiata* L.), it was found that there was a concentration-dependent decrease in root nitrate reductase. Leaf nitrate reductase, on the other hand, increased significantly with increasing Pb concentration (Singh *et al.*, 1997).

6.2.2 Electron microscopy

With transmission electron microscopy, in addition to elucidating ultrastructure, it is possible to identify elemental composition through electron probe microanalysis, and localise electron dense deposits, such as lead, in biological substances. Materials examined in this manner include algae (Silverberg, 1975), the aquatic plant species *Potamogeton pectinatus* (Sharpe and Denny, 1976), radish (Lane and Martin, 1982), the grass *Anthoxanthum odoratum* (Qureshi *et al.*, 1986), and tissue cultures of *Populus* sp. (Ksiazek *et al.*, 1984). In all cases, Pb was found in high levels in the cell walls, and in the tissue cultured cells it was also found in intercellular spaces, vacuoles, and dictyosomes. Similar studies undertaken on Pb on lupin roots (Przymusinski and Wozny, 1985), showed that growth was inhibited, roots became thicker, and the central region of the meristem seemed to be protected from Pb. Various aspects of Pb uptake and accumulation by onions (*Allium cepa* L.) have been examined by transmission electron microscopy including: Pb^{2+} migration through root tissues (Wierzbicka, 1987); constitutional tolerance to Pb (Wierzbicka, 1995); Pb-treated roots

(Wierzbicka, 1998); and a report establishing how much Pb is removed from the root tips of onion during the successive stages of fixing and dehydrating in preparation for electron microscopy (Antosiewicz and Wierzbicka, 1999).

Approximately 4% of the Pb is lost during chemical preparation indicating that conventional electron microscopy is suitable for studying the distribution of Pb in *A. cepa* root tip cells. Other related work by the same group, on onions, included a study of the effects of Pb on root cell division (Wierzbicka, 1994), and the differences in tolerance to Pb between onion plants developing from seeds and bulbs (Michalak and Wierzbicka, 1998).

6.2.3 Mineral status

The relationship between constitutional tolerance to Pb and mineral status was examined in four crop species; mustard, flax, tomato and sunflower (Antosiewicz, 1993). Tolerance to deficit of P, Ca and Mg, and to general nutrient stress was also reported. In a similar study undertaken on two metal tolerant species, *Silene inflata* and *Biscutella laevigata*, possible relationships between constitutional and inducible Pb-tolerance and tolerance to mineral deficits were examined (Antosiewicz, 1995).

6.3 Forest species

Some environmentally based studies have sought to quantify trace metal accumulation, including Pb, *in vivo,* in conifers such as Norway spruce (*Picea abies*) (Krivan and Schaldach, 1985). Karandinos *et al.* (1985), sampled bark and wood from Aleppo pine (*Pinus halepensis*) trees to assess Pb pollution in the Greater Athens Region. Other work on conifers has included investigations into effects of Pb on mycorrhizal associations with Norway spruce (*Picea abies*) (Jentschke *et al.*, 1991). In another study, spruce seedlings inoculated with natural mycobionts were grown in natural forest humus containing Pb from atmospheric inputs (Jentschke *et al.*, 1997).

More recently this group extended the scope of this work by including the N source, and thus pH, into the equation regarding Pb uptake by *P. abies* seedlings (Jentschke *et al.*, 1998). European beech (*Fagus sylvatica* L.) has also been studied in relation to trace metal uptake and physiological effects (Breckle and Kahle, 1992).

(7) PHYTOREMEDIATION AND LEAD

7.1 Rhizofiltration

The earliest rhizofiltration study on Pb, was conducted on 4-5 week-old Indian mustard (*Brassica juncea* L.) plants grown hydroponically (Dushenkov *et al.*, 1995). Primarily Pb removal from aqueous solution and root accumulation was investigated but Cd, Cu, Cr, Ni and Zn were also included. It was found that *B. juncea* roots concentrated Pb 563-fold above initial solution concentrations and that the roots of many hydroponically grown terrestrial plants could effectively remove toxic metals from aqueous solutions. Pb removal was based on tissue absorption and on root-mediated Pb precipitation in the form of insoluble inorganic compounds, mainly $Pb_3(PO_4)_2$. In a later study, seedlings of *B. juncea* grown in the dark, in aerated water (aquacultured), were able to accumulate various metals, including Pb, from artificially contaminated water over a range of metal concentrations in the presence of the competing ions Ca, Mg, K, SO_4, and NO_3 (Salt *et al.*, 1997).

7.2 Phytoextraction of Pb

In the first lead phytoextraction study, the ability of various crop plants to accumulate Pb in shoots and roots, in sand culture, was compared (Kumar *et al.*, 1995). All crop Brassicas accumulated Pb but cultivars of *B. juncea* (L.) Czern. showed a strong ability to accumulate Pb in roots and to transport it to the shoots.

B. juncea was also able to concentrate Cr^{6+}, Cd, Ni, Zn, and Cu in the shoots from a substrate containing sulphates and phosphates as fertilisers.

In a study on Pb transport comparing corn (*Zea mays* L. cv Fiesta) with ragweed (*Ambrosia artemisiifolia* L.), both accumulation and physiological aspects were characterised (Huang and Cunningham, 1996). In solution culture, ragweed (dicot.) accumulated significantly more Pb in roots than corn (monocot.), and corn accumulated significantly more Pb in shoots. Pb exposure decreased dry weights of roots and shoots, and inhibited root elongation and lateral root initiation. Comparisons for phytoextraction efficiency with known metal accumulators were also conducted. Of the 11 species/cultivars tested using both nutrient solutions and contaminated soils, corn accumulated the highest shoot-Pb concentration. Addition of N-(2-hydroxyethyl) ethylenediamine triacetic acid (H-EDTA) at 2.0 g kg^{-1} to contaminated soil resulted in a surge of Pb accumulation in corn, from 40 mg kg^{-1}(-H-EDTA) to 10,600 mg kg^{-1} (+H-EDTA). Desorption trials showed that H-EDTA was effective in desorbing apoplastic lead from roots.

Chelate addition to Pb-contaminated soils to increase Pb accumulation in plants was investigated in a study on corn (*Zea mays* L. cv Fiesta), pea (*Pisum sativum* L. cv Sparkle), goldenrod (*Solidago bicolor* L.) and sunflower (*Helianthus annuus* L. cv Avante). The addition of chelates to a Pb-contaminated soil (total soil Pb 2500 mg kg^{-1}) increased shoot concentrations of corn and pea from less than 500 mg kg^{-1} to more than 10,000 mg kg^{-1}. Five different chelates were assessed for their ability to enhance Pb levels in the soil solution and to increase Pb translocation from root to shoot. Ethylenediaminetetraacetic acid (EDTA) was found to significantly increase Pb translocation from root to shoot (Huang *et al.*, 1997). In a similar study, it was demonstrated that accumulation of five toxic metals, including Pb, by *B. juncea* could be enhanced with synthetic chelates (Blaylock *et al.*, 1997). Concentrations of 1.5% Pb in the shoots of *B. juncea* were obtained from soils containing 600 mg Pb kg^{-1} amended with synthetic chelates such as EDTA. EDTA-enhanced accumulation of Pb was further promoted by lowering the pH of the substrate.

Vassil *et al.* (1998) demonstrated that a threshold concentration of 0.25 mM EDTA was required to stimulate the accumulation of 1.1% Pb in dry shoot

tissue of *B. juncea* representing a 75-fold concentration of Pb over that in solution. Analysis of xylem exudate confirmed that the majority of Pb in these plants is transported as a Pb-EDTA complex. The accumulation of EDTA in shoot tissue was directly correlated with the accumulation of Pb in shoot tissue. In a trial examining the effects of varying concentrations of Pb on seed germination, root, and shoot length in *Brassica pekinensis* cv JF-1 (Xiong, 1998), a general concentration-dependent inhibition of all parameters was observed, with seed germination unaffected at 125 mg Pb L^{-1} and falling to 43% at 1000 mg Pb L^{-1}.

A theoretical approach to phytoextraction was the basis of the work done by Brennan and Shelley (1999) in attempting to model the fundamental processes involved in Pb uptake and transport. Utilising previously published data, (Huang and Cunningham, 1996; Huang *et al.*, 1997; Blaylock *et al.*, 1997) they developed a mechanistic system dynamics model that simulated extraction and translocation of Pb by a maize plant. Results of model simulations suggest that precipitation of Pb as a $Pb_3(PO_4)_2$ complex is one of the most important mechanisms for transport within the plant.

(8) PLANT IMPROVEMENT

8.1 Transgenics

Creating plants that can tolerate and/or sequester toxic trace metals in non-consumed tissues has been a goal in agriculturally important species such as *Brassica napus* for some time. One approach to this involved creating a chimeric gene containing human metallothionein-II and introducing it into *B. napus* and *Nicotiana tabacum* cells in tissue culture, via *Agrobacterium tumefaciens* (Misra and Gedamu, 1989). Transgenic seedlings derived from regenerated transformants were unaffected by 100 μM $CdCl_2$ in the growth medium, whereas control seedlings showed severe inhibition of growth and chlorosis of leaves.

A similar approach was used in inserting a yeast metallothionein gene into tobacco plants with *Agrobacterium* (Truksa *et al.*, 1996). Selected transformants

from the F_1 generation displayed higher tolerance to toxic metals in the early stages of development and the preliminary results of metal uptake experiments showed increased capacity for metal accumulation.

Another approach using *Arabidopsis thaliana* involved transforming plants with a modified bacterial gene, *merBpe*, encoding organomercurial lyase, which allows growth on normally lethal concentrations of phenylmercuric acetate or methyl-mercuric chloride and sequestration of Hg (II) (Bizily *et al.*, 1999).

8.2 Mutants

Mutant plants may be created using mutagenic agents such as ethyl methanesulphonate (EMS) and then screening the progeny for desirable traits. This was accomplished in *A. thaliana* ecotype Colombia where mutagenised seedlings with abnormal count rates for Mn were identified by x-ray fluorescence spectrometry, and grown to produce seed (Delhaize, 1996). One seedling that showed an inherited abnormality was further studied and found to accumulate a range of metals including Mn, Cu, Zn, and Mg in leaves, to much higher levels than the wild type.

EMS was also used in the development of a new screening method for rapidly identifying mutants capable of accumulating large amounts of toxic trace metals (Schulman *et al.*, 1999). *B. juncea* (L.) Czern seedlings containing radioisotopes of the metals of interest were visualised with a phosphorimager. Mutants were recovered that retained increased accumulation through the third generation. One mutant in particular contained more root cell wall material on a fresh weight basis than wild types and perhaps as a result of this, displayed enhanced Pb accumulation.

8.2.1 Transpiration mutants

There is a potential efficiency gain in phytoextraction by enhancing plant transpiration. It has been demonstrated that wind enhances metal flux to the shoots, while compounds that block transpiration (i.e., abscisic acid) block metal

accumulation in the shoots (Salt *et al.*, 1995a). Seedlings grown from *B. juncea* seeds mutagenised with EMS, were screened by assessing tissue dehydration after 1 or 2 hours in a well aerated room (Gleba *et al.*, 1999). Plants with increased transpiration rates were identified. One line exceeded the transpiration rate of the wild-type plants by 130% in soil and 75% in hydroponics. In a Pb-contaminated soil, amended with EDTA, the high transpiration line extracted 104% more Pb than the wild-type *B. juncea* making it a good candidate for field optimisation and use.

8.3 Acclimation

Acclimation is defined as the gradual and reversible adjustment of physiology and morphology to changes in environmental conditions. Studies examining phenotypic plasticity, or the plant's innate response to environmental stress, have been conducted. In a study investigating acclimation to metal stress by Cu, Cd and Zn, of various *Salix* species and clones, short-term pre-treatments and gradual acclimation to elevated metal levels were compared (Punshon and Dickinson, 1997). It was found that gradual cumulative doses resulted in reduced phytotoxicity and increased resistance, particularly to Cd, and was more effective than a short-term pre-treatment.

(9) TISSUE CULTURE

Plant tissue culture broadly refers to the *in vitro* cultivation of any plant part, whether single cells, tissues, or organs, under aseptic conditions (Thorpe, 1981). Tissue culture is based on the concept of totipotency which means that, in theory, differentiated plant cells retain the genetic information required for the development of a complete plant. A tissue culture cycle involves the establishment of a more or less dedifferentiated cell or tissue culture under defined conditions, proliferation for a number of cell generations and, in theory,

the subsequent regeneration of whole plants (Larkin and Scowcroft, 1981). The ease with which this can be achieved varies enormously from one species to another.

The initiating explants for a tissue culture cycle may come from virtually any plant organ or cell type including embryos, microspores, roots, leaves and protoplasts. Aseptic plant tissue cultures consist mainly of embryo cultures, organ cultures, callus cultures, suspension cultures, or whole plant cultures (Teutonico and Knorr, 1984). The most extensive application of plant tissue culture in agricultural production has been rapid *in vitro* propagation, also known as clonal propagation, which involves asexually reproducing plants of uniform quality in large quantities (Murashige, 1978).

9.1 Somaclonal variation

The use of tissue culture for clonal propagation is based on the assumption that tissues remain genetically stable when excised from the parent plant and placed into culture. This assumption is largely valid when plant multiplication occurs by development of axillary buds or adventitious shoots, directly from explanted organs. However, when shoot formation is induced from callus tissues, aberrant plants are often produced, and the frequency of such aberrant types increases with the length of time the callus is maintained *in vitro* (Chaleff, 1983).

That genetic variability arises spontaneously in plant cell cultures has been confirmed by several lines of evidence including karyotypic variation (polyploidy, aneuploidy, and chromosomal rearrangements) and novel cellular phenotypes such as spontaneous co-resistance to picloram and hydroxyurea (Chaleff, 1983). No adequate explanation has ever been advanced for the phenomenon of culture variation or the mechanisms by which it occurs. It may be the culture environment itself is mutagenic, since stable variants arise from it (Larkin and Scowcroft, 1981). It may result from the breakdown of normal cellular or mitotic processes or from the activation of genetic systems, such as transposable elements that are normally repressed.

Another possibility is that such aberrant events occur at the same frequency in the intact plant but that selection against them is less stringent *in vitro* than *in vivo* (Chaleff, 1983). The term 'somaclone' has been proposed for plants derived from any form of cell culture (Larkin and Scowcroft, 1981) and 'somaclonal variation' is now commonly used to describe the variation displayed amongst such plants and the cells from which they arise.

9.2 *In vitro* selection

One advantage of plant cell/tissue culture is the ability to directly select for novel phenotypes from large physiologically and developmentally uniform populations of cells grown under defined conditions. Incorporation of toxic or inhibitory substances in the medium allows growth only of the few resistant cells in the population, and from these isolates, plants can ultimately be regenerated, in theory. The existence of a gradient of susceptibility to the selection agent may be induced by an exogenous source such as a chemical mutagen, or it may be innately present through somaclonal variation. There are, however, several constraints to *in vitro* selection such as, not all traits expressed by the whole plant are expressed by the cultured cell, e.g., drought tolerance. Conversely, not all traits expressed by the cultured cell are expressed by the whole plant. Some traits though, such as salt tolerance, can be affected by several different mechanisms- some acting at the cellular level and others at the whole plant level. An example of this is a study in which alfalfa (*Medicago sativa* L.) plants regenerated from salt-tolerant cell lines showed improved salt tolerance compared to parent plants (Winicov, 1991).

Since the resistance of plants to metal stress involves cellular adaptations to ion toxicity, it is conceivable that *in vitro* techniques may be effective in selecting or screening for desirable characteristics such as tolerance, uptake, phytochelatin production, or compartmentalisation. It is important however, to establish a correlation between cellular and whole plant responses to metal stress, and whether genotypic differences in resistance can be expressed in cultured cells

(Petolino and Collins, 1984). Callus and whole plant responses to Cu and Zn toxicity have been reported (Wu and Antonovics, 1978). Callus derived from a genotype of *Agrostis stolonifera* which was tolerant to both Cu and Zn, when exposed to 1 ppm Cu and 12 ppm Zn, increased its dry weight to about 5 times that of a non-tolerant genotype. Plants regenerated from callus of the tolerant genotype maintained higher Cu and Zn tolerance than plants regenerated from callus derived from the non-tolerant genotype. Suspension cultures have also been used to select cells for Cd resistance in studies such as Jackson *et al.* (1984) where *Datura innoxia* cells were selected, and Huang *et al.* (1988) where tobacco cells resistant to Cd were studied in relation to temperature stress. However, in neither case were plants regenerated from the selected cell lines.

Ultimately, for *in vitro* selection to be viable, the tissue culture protocols must be well developed for the species in question, the trait to be modified must be expressed in tissue culture, effective selection of the desired variant must be possible, and probably most important of all, variant cell lines must be capable of regenerating plants in which the selected trait is expressed, stable, and heritable in succeeding generations.

9.3 Somatic hybrids

Somatic hybridisation, a relatively novel tissue culture technique, was carried out in an attempt to introduce metal resistant traits into *B. juncea* because although it exhibits a high capacity for metal uptake and translocation, it is not very resistant to high levels of Pb and other toxic metals in its foliage. *T. caerulescens* was selected as one of the parents for both symmetric and assymetric hybrids in which protoplasts were irradiated with x-rays before fusion. Two assymetric hybrids were found to be fertile and one of these displayed increased resistance when grown on Pb, Ni, and Zn contaminated soil. Pb extracted on a dry weight basis was similar to both parents but the total amount extracted was greater due to the increased biomass produced on the contaminated soil (Gleba *et al.*, 1999).

(10) PINUS RADIATA

Monterey pine (*Pinus radiata* D. Don) is a three-needled pine that evolved in California on nutrient poor soils where its growth habit is unspectacular and the species is not of any significant commercial value. In New Zealand it has an exceptionally fast growth rate and is the principal forestry species (Rimbawanto *et al.*, 1988), with 1.2 million hectares planted (Devey *et al.*, 1996). In ideal situations it can add 1m of growth per year and be ready for milling in 25 years.

Traditionally *P. radiata* in New Zealand has been propagated by seed from superior stock, wind pollinated, in clonal seed orchards. Early on, however, clonal vegetative propagation was recognised as a means of capitalising on outstanding genotypes while avoiding genetic segregation and the consequent variability that arises with propagation by seed (Smith, 1986). As a consequence, techniques were developed to produce rooted cuttings and air layers of radiata pine (Cameron and Thomson, 1969) and more recently it was demonstrated that whole plantlets could be regenerated from radiata pine embryos and cotyledons by micro-propagation (Reilly and Washer, 1977).

(11) TREE LUCERNE

Tagasaste, or tree lucerne (*Chamaecytisus proliferus* (L.f.) Link ssp. *proliferus* var. *palmensis* (H.Christ.)), originated on the island of La Palma in the Canary Islands but has achieved some recognition beyond the boundaries of these islands and is considered to have high potential for agroforestry systems of semiarid regions of the subtropics. It has become a popular crop in New Zealand since it was introduced in the late 19th century (Sanchez-Yelamo *et al.*, 1995).

Tree lucerne is a fast growing, evergreen, leguminous shrub or small tree which is relatively easy to propagate, establish from seed, and grows well on nutrient poor soils. Growth in excess of 1m per year is quite common with a terminal height of about 10m after 5 to 10 years. Typically tree lucerne is characterised in the literature as a fodder-crop species and the majority of

publications relate to this aspect of its culture (Gonzalez-Andres and Ortiz, 1996). Some work, involving electrophoresis of seed proteins, was carried out on its taxonomic status (Sanchez-Yelamo *et al.*, 1995). In New Zealand, the plant's habit of spreading on roadside verges and uncultivated land has led to it being labelled as a weed by some but the ease with which it is able to colonise barren, low-nutrient soils has not gone unnoticed (Fitzgerald, 1982).

(12) OBJECTIVES OF THIS STUDY

The purpose of this study was primarily to determine if *P. radiata* and/or *C. palmensis* were potentially suitable candidates for a programme of selection and screening aimed at improving their capacity for Pb phytoextraction within the general framework of phytoremediation. As they were not previously regarded as phytoextractors, it was important to characterise aspects of their performance in the role of trace metal phytoextraction, such as tolerance, uptake, and translocation, and compare them with each other and other acknowledged phytoextraction species. In addition, the intention was to examine mechanisms that impeded and promoted the process, and also consider options for improving the plants efficiency in metal extraction. We decided to address one of the main problems in the field, that of low biomass accumulation, right from the outset, by selecting fast growing trees that accumulate significant biomass. To date, trees are not well represented or characterised within the phytoextraction literature, with a few notable exceptions such as *Pinus sp.*(Entry *et al.*, 1993) and *Salix sp* (Punshon and Dickinson, 1997).

The choice of one angiosperm and one gymnosperm was deliberate to provide a basis for comparison. The conifer, *P. radiata,* is reasonably well characterised in many aspects of its growth and propagation, both in the field and in tissue culture, and has some history in phytoremediation (Entry *et al.*, 1993). The legume, *C. palmensis,* is unknown in this type of research but has many desirable attributes such as fast growth, ease of establishment, is non-deciduous and has a propensity to grow on marginal soils.

Pb was chosen as the contaminant of interest as it is widely acknowledged as one of the most widespread and difficult metal pollutants to remediate effectively (Brennan and Shelley, 1999; Kumar *et al.*, 1995), and if species demonstrate ability to extract Pb, it is entirely possible that they may also be able to eventually extract other less recalcitrant trace metal contaminants, such as Cd.

Initially, techniques needed to be developed to provide continuous stocks of resource materials for experimental manipulations. As trees are relatively slow growing and many analytical techniques are destructive, significant volumes of plants were required. To reduce uncertainty within the results, we decided to work with clonal materials as soon as was practicable. In the meantime, studies on seedlings were commenced to determine baseline data for comparative purposes.

Early on, both species were inducted into tissue culture, initially to produce clones but also to gauge amenability to *in vitro* selection/screening. *P. radiata* was assessed for its ability to survive as long term subculturable meristematic tissue. *C. palmensis* was exposed to media containing a variety of different plant growth regulators in attempts to induce shoot organogenesis for the purpose of regenerating whole plants.

Effects of Pb on different aspects of plant growth were examined such as inhibition of seed germination, root growth inhibition, fresh weight changes of *in vitro* tissue, and responses of seedlings and excised shoots in solution culture.

Pb uptake studies using solution culture were undertaken at various concentrations with and without the addition of the chelators H-EDTA and EDTA. Quantitative analysis of both root and shoot tissue, for seedlings and selected clones, was conducted using flame atomic absorption spectrometry.

Transmission electron microscopy was carried out on tissue from both *P. radiata* and *C. palmensis* exposed to Pb, with and without the addition of chelators, to localise the deposition of electron-dense deposits of Pb at the cellular level and to compare these findings with the data from the Pb uptake trials.

Phosphate and Pb are known to precipitate together in solution and aspects of this were investigated including changes in acid phosphatase activity within Pb-exposed tissues, and the presence and/or changes of acid phosphatase isozyme banding patterns in response to Pb exposure.

12.1 Summary of the aims of this study

(1) To examine the phytoremediation potential of *P. radiata* and *C. palmensis*, both as seedlings and clones, in extracting and accumulating Pb.

(2) To characterise physiological responses of plants to Pb exposure including inhibition of seed germination, root growth inhibition, and *in vitro* responses.

(3) To assess the ability of *P. radiata* and *C. palmensis* as candidates for *in vitro* selection/screening to improve their Pb phytoextraction capabilities.

(4) To localise Pb deposition and translocation within the tissues of the plants, both between root and shoot, and at the cellular level.

(5) To examine aspects of the role of P in Pb extraction particularly with regard to acid phosphatase activity in response to Pb exposure.

CHAPTER II

MATERIALS AND METHODS

(1) PLANT PRODUCTION

1.1 Seeds

Pinus radiata (D.Don) seeds, (seed treatment S 14) were obtained from New Zealand Tree Seeds, Rangiora. All *P. radiata* seeds were stored at 4° C.

Mature seeds of *Chamaecytisus proliferus* (L.f.) Link ssp. *proliferus* var. *palmensis* (H.Christ.) were obtained from open pollinated trees in Ruby Bay, Nelson, and stored at room temperature.

1.2 Seed germination (non-aseptic)

P. radiata seeds were placed on one sheet of Whatman No.1 filter paper in a 90 mm plastic Petri dish, covered with 10 ml dH_2O, and kept at 4° C, in the dark, for 14 days for moist chilling (stratification). After this, the seeds were moved to 26° C in a dark growth room for germination to proceed. The first visible sign of seed germination appeared after approx. 5 days. A *P. radiata* seed is considered germinated when the radicle length has reached four times that of the seed (Rimbawanto *et al.*, 1988).

C. palmensis seeds were placed in a tea strainer in boiling water for 60 seconds to break seed coat-imposed dormancy. Then the seeds were germinated in the dark growth room as described above. The first visible sign of germination appeared after approximately 14 days. When the emerging radicle has reached 3 mm in length, the seed is considered to have germinated (Gonzalez-Andres & Ortiz, 1996).

1.2.1 Immature *C. palmensis* seed germination

Immature seeds were obtained from wild populations of *C. palmensis* trees growing in Wainui, Akaroa Harbour. The seeds were examined for size, colour, and developmental stage of the embryo in relation to germinability. Differences were noted in stages of maturity. Some immature embryos, isolated from all seed structures, were tested directly for germination on moist filter paper in Petri dishes at 22° C in continuous light, while some were partially desiccated, rehydrated and then tested for germination (embryo rescue).

1.3 Seedlings

Although it was decided from the outset to work with clonal materials as soon as practicable, the time lag in producing them was significant. Approximately 24-30 months elapsed before any quantities of clonal materials were available. This coupled with the large volume of plant material required for destructive analyses necessitated that approximately 50-100 seedlings each of *P. radiata* and *C. palmensis*, at various sizes, be maintained under standard glasshouse conditions (ambient light, 18-22° C). In addition small quantities, approximately 10-20, of very small seedlings, approximately 10 cm height, of both species were maintained in modified Hoaglands nutrient solution (Hopkins, 1995) in McCartney bottles in 16 hour light at 22° C, as required for small scale studies on lead effects, lead uptake, and enzyme visualisation.

1.4 Soil-less growth media (sand culture)

All seedlings and clones maintained under non-aseptic glasshouse conditions were grown in a soil-less growth medium that comprised perlite (Hortlink Ltd., Christchurch), sharp sand (collected from Birdling's Flat, Christchurch), and sphagnum peat moss (Australasian Peat Ltd.) mixed together in a ratio of 2:2:1 and fertilised with Osmocote Plus (Grace Sierra Austr. Pty. Ltd.) at

the rate of 1.5-3.5 kg m^{-3}. Vegetative propagation of *P. radiata* shoot cuttings and fascicles was carried out in a propagation medium comprising sharp sand, perlite, and sphagnum peat moss mixed together in a ratio of 6:3:1. Seedlings grown under aseptic conditions were grown in vermiculite (Nuplex Industries Ltd., Auckland) that was autoclaved at 121° C, 1.1 kg cm^{-2}, for 20 minutes.

1.5 Propagation of Forest Research Institute clones

Vegetative propagation was carried out on the set of Forest Research Institute (FRI) *P. radiata* clones designated Q, R, S, T, U, V, W, X, Y, and Z, maintained in the Canterbury University glasshouse complex. Groups of fascicles and uniform terminal shoot cuttings were taken, moistened with water, and dipped in semi-hardwood Seradix powder (Rhone-Poulenc N.Z. Ltd., Lower Hutt) containing 1g kg^{-1} β-indolylbutyric acid (IBA). The cuttings/fascicles were placed 25 mm deep in propagation medium in propagation trays, under intermittent mist, in the glasshouse, at approximately 18-22° C. Rooting was assessed by carefully removing cuttings/fascicles from the medium, visually inspecting for callus or root formation before returning them to the medium. When roots formed, the plants were removed from the mist, transferred to the same growth medium in 50 mm plastic pots, and placed under standard glasshouse conditions (ambient light, 18-22° C).

1.6 Seed germination (aseptic)

Viable *P. radiata* seeds (uniform colour and shape), were placed in a tea strainer and immersed in 80 ml of solution containing 2.5% available Cl in dH_2O diluted from a commercial bleach containing 4.8 ± 0.2 % sodium hypochlorite (NaOCl) for 15 minutes. Seeds were then rinsed in running water for approx. 2 hours, placed on one sheet of Whatman No.1 filter paper in a 90 mm plastic Petri dish, covered with 10 ml dH_2O, and kept at 4° C, in the dark, for 14 days for moist

chilling (stratification). After this the seeds were sterilised again in 2.5% available Cl for 15 minutes with occasional stirring. Then they were rinsed 3 times in sterile dH_2O in the laminar flow hood (Airpure Email Westinghouse Pty. Ltd., N.S.W., Aust.) after which they were transferred aseptically, either into sterilised 250 ml polycarbonate tissue culture vessels (approximately 60 mm x 80 mm) containing 20 mm depth of sterile vermiculite and 20 ml of sterile dH_2O, or sterile Petri dishes containing a filter paper and 10 ml of sterile dH_2O. These containers were moved to a dark growth room at 26° C.

Viable *C. palmensis* seeds (uniform colour and shape), were placed in a tea strainer in boiling water for 60 seconds to break seed coat dormancy. Then they were placed in 80 ml bleach solution containing 2.5% available Cl in dH_2O, with 2 drops of Tween 20 (BDH Ltd., Poole, England) added, for 15 minutes, with occasional stirring. Then, in the laminar flow hood they were rinsed 3 times in sterile dH_2O and transferred aseptically to either sterile vermiculite in 250 ml tissue culture vessels or sealed Petri dishes containing a filter paper and 10 ml of sterile dH_2O. The containers were kept in the dark growth room at 26° C. After the majority of seeds had fully imbibed, typically 1-2 days, excess moisture was carefully drained from the Petri dish without removing the lid. This moisture reduction step is very important in preventing microbial growth on the seeds.

(2) TISSUE CULTURE

2.1 *P. radiata* culture media

The culture medium for the generation of meristematic tissue and initiation of adventitious shoots on *P. radiata* cotyledons consisted of modified Quoirin and LePoivre medium (Aitken-Christie *et* al., 1988) containing major and minor salts, vitamins and Fe (appendix A). To this was added myo-inositol (Sigma) at 1g L^{-1}, sucrose (Chelsea, N.Z. Sugar Co. Ltd., N.Z.) at 30 g L^{-1}, benzyladenine (BA), (Sigma) at 22.19 μM and agar (Germantown N.Z. Co., N.Z.) at 8 g L^{-1}. This medium was designated LP5. Shoot elongation medium was identical to LP5

without BA. This medium was designated LPO. The pH of all media used in *P. radiata* tissue culture was adjusted (pH meter E350B, Metrohm Herisau, Switzerland) to between 5.6 and 5.8 with either 1M NaOH or 1M HCl, prior to autoclaving as in 1.4.

2.1.1 Cotyledon culture (a) meristematic tissue generation

Stratified seeds that were aseptically surface sterilised were allowed to germinate at 26° in the dark for 5-7 days to allow the radicle to emerge to between 0.5 and 2.0 cm. The seed coat and megagametophyte were then removed aseptically in the laminar flow hood and the cotyledons were excised. Each seed had between 7-10 cotyledons that responded uniformly when cultured (Aitken-Christie & Thorpe, 1984). In some cases, cotyledons were additionally surface sterilised aseptically in 2.5 % available Cl in dH_2O for 5 minutes, followed by three rinses in sterile dH_2O, due to sporadic contamination. The tips of the cotyledons were removed aseptically to prevent elongation, the remainder were trimmed to approximately 3mm and placed horizontally on sterile LP5 medium, in 90mm plastic Petri dishes. Cultures were placed under continuous cool white fluorescent light at an average intensity of approximately 150 $\mu mol\ m^{-2}\ s^{-1}$ (Li-Cor, LI-185A photometer Evans Electroselenium Ltd., Essex, England) at 22° C.

2.1.2 Cotyledon culture (b) shoot initiation and elongation

After meristematic tissue formed and shoot initiation was evident on the cotyledonary explants, which typically took about three to four weeks, they were transferred aseptically to sterile shoot elongation medium in 90 mm plastic Petri dishes, and returned to continuous light at 22° C. When shoots had elongated sufficiently (approximately 5-10 mm), they were transferred aseptically to 250 ml tissue culture vessels containing 40 ml sterile elongation medium. Once established, shoots were subcultured aseptically approximately every 8-10 weeks, depending on growth rates, which varied considerably among the clones. Subculturing involved cutting sections from main shoots and splitting up clumps of shoots, aiming to end up with 4-6 shoots per 250 ml tissue culture jar.

2.1.3 Cotyledon culture (c) long-term subculturable meristematic tissue

Cotyledons derived from surface sterilised seeds were surface sterilised aseptically either in 2.5% available Cl for 5 minutes or 100 % ethanol for 10 seconds. These were placed aseptically on LP5 in 90 mm plastic Petri dishes. Subsequently meristematic tissue excised from these plates was subcultured on half-strength LP medium (Bergman and Stomp, 1992) in shallow Petri dishes containing 11.10 μM BA, 30 g L^{-1} sucrose, and 8 g L^{-1} agar. This medium was designated 1/2 LP 2.5. To overcome contamination, as tissue was subcultured it was aseptically surface sterilised as necessary, with 2.5 % available Cl in dH_2O for 10 minutes followed by three rinses of sterile dH_2O. In addition, Plant Preservative Mixture (PPM), (Austratec Pty. Ltd.) was incorporated into the medium at 1.0 ml L^{-1}.

2.1.4 *P. radiata* clonal root induction

All root induction and root elongation trials were conducted in a growth room with 16 h light daily at 50 μmol m^{-2} s^{-1} at 22° C. Initially attempts were made to induce roots on *in vitro* clones by aseptically transferring batches of 5 single shoots with 30-50 mm axes (Horgan and Aitken, 1981), to separate 50 ml tissue culture vessels containing approximately 16 ml of water agar comprising 8 g L^{-1} agar, 30 g L^{-1} sucrose, and no nutrients. This was later modified not to include sucrose. Subsequently a shoot preconditioning treatment (Smith, 1986) was included that utilised root induction medium (RIM), consisting of LPO plus 9.84 μM indol-3-ylbutyric acid (IBA), (BDH) and 2.69 μM α-naphthalene acetic acid (NAA), (Sigma) (Reilly and Washer, 1977), and 20 g L^{-1} sucrose. *In vitro* shoots with 15-30 mm axes were preconditioned on RIM for 10 days before they were transferred aseptically to root elongation medium (REM), consisting of LPO plus 20 g L^{-1} sucrose. The preconditioning step was first increased to at least 12 days (Rancillac *et al.*, 1982). In later attempts, NAA was omitted and IBA was trialed at the concentrations of 9.84, 24.60, 49.20, 73.80, and 98.40 μM. Also, the preconditioning step was lengthened to at least 14 days, and batch sizes were increased to 10-20 shoots. Eventually, the IBA concentration was lowered to either 2.46 or 4.92 μM for all preconditioning treatments, and instead of

transferring preconditioned shoots to REM, they were placed directly into a mixture of sharp sand, perlite and peat mixed together in a ratio of 2:2:1, in small seedling trays, under *ex vitro* conditions. These were covered with clear plastic lids to control humidity and fungal pathogens were controlled with Thiram (tetra-methyl-thiuram disulphide.) (Monsanto) at 1.5 g L^{-1}. Rooting was assessed by removing shoots from soil-less mix at seven day intervals, examining shoots for callus and root initiation before returning them to the mix. Rooted shoots were transferred to soil-less media with humidity control, gradually hardened off by allowing more gas exchange, and eventually transferred to standard glasshouse conditions.

2.2 *C. palmensis* culture media

Basal medium consisted of half strength Murashige and Skoog (1962) salts, half strength iron stock, half strength organic stock, half strength vitamin supplements, sucrose at 30 g L^{-1}, agar at 8 g L^{-1} and is hereafter referred to as 1/2 MS. In all cases pH of the media was adjusted to between 5.6 and 5.8 with either 1M NaOH or 1M HCl prior to autoclaving. In addition, supplemented media used in callus induction and organogenesis trials included:

- 1/2 MS plus 2,4-dichloro-phenoxy-acetic acid (2,4-D), (BDH) at 10 μM
- 1/2 MS plus 5 μM BA
- 1/2 MS plus factorial combinations of 2,4-D at 0, 2.26, 4.52, 9.05, 22.62, and 45.24 μM and BA at 0, 2.22, 4.44, 8.88, 22.2, 44.4 μM
- 1/2 MS plus 22.2 μM BA (designated D5, 0.0)
- 1/2 MS plus semi-factorial combinations of BA at 0, 11.1, and 22.2 μM and NAA at 0, 13.43, and 26.85 μM
- 1/2 MS plus 4.54 μM 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (TDZ), (Sigma) and 2.07 μM (4-amino-3,5,6-trichloropicolinic acid) (picloram), (Sigma) (designated M1,0.5)
- 1/2 MS plus 4.54 μM TDZ and 4.14 μM picloram (designated M1,1)

- 1/2 MS plus 4.54 μM TDZ (designated M1)
- 1/2 MS (90 g L^{-1} sucrose) plus 10% (v/v) coconut water (Sigma) and 10 μM 2,4-D (designated M$)
- 1/2 MS (90 g L^{-1} sucrose) with no supplements designated media (A), plus 4.52 μM 2,4-D designated media (B), plus 4.14 μM picloram designated media (C), plus 9.04 μM 2,4-D designated media (D). Callus from (A), (B), (C), or (D) was transferred to (1) Full strength MS (100 g L^{-1} sucrose) plus 9.04 μM 2,4-D plus 10 % (v/v) coconut water designated C2.0, (2) Full strength MS (100 g L^{-1} sucrose) plus 13.57 μM 2,4-D plus 5 % (v/v) coconut water designated C3.3.

2.2.1 *C. palmensis* callus induction

a) Initial explants were surface sterilised cotyledons, hypocotyls, and roots (derived from aseptic seeds 7 days after germination), cultured aseptically in Petri dishes, containing 20 ml sterile 1/2 MS, with and without 10 μM 2,4-D, at 22° C in continuous light (as were all other callus and organogenesis induction attempts unless otherwise specified). At this stage a red pathogen began to infect a significant proportion of all plates and various methods were trialed to eradicate it starting with dipping explant material briefly in 100% ethanol. (refer to results section).

b) Surface sterilised embryos derived from surface sterilised immature seed were aseptically cultured on 20 ml sterile 1/2 MS plus 10 μM 2,4-D.

c) A factorial plant growth regulator trial was undertaken with surface sterilised cotyledons and shoot apices, using 20 ml sterile 1/2 MS supplemented with 2,4-D at 0, 2.26, 4.52, 9.04, 22.62, or 45.24 μM and BA at 0, 2.22, 4.44, 8.88, 22.2, or 44.4 μM. Cotyledons and shoot apices of mature embryos were bisected longitudinally and placed flat on the medium, in Petri dishes, which were completely randomised, for up to 35 days.

2.2.2.1 Shoot organogenesis induction trials:Media transfers

a) Axenic callus derived from roots, immature seeds, cotyledons, and hypocotyls was transferred from 1/2 MS plus 10 μM 2,4-D, to 1/2 MS plus 5 μM BA in an attempt to induce shoot formation from the callus cultures. Large clumps of callus (10-12 mm diameter) were cut into small pieces (4-5 mm diam.) and placed firmly in contact with the medium.

b) In an attempt to regenerate shoots, callus derived from media containing various plant growth regulator concentrations was transferred to 1/2 MS plus 22.2 μM BA (D5, 0.0). Large pieces of callus were aseptically cut into smaller pieces and placed in close contact with the fresh medium.

c) Callus from various origins e.g., subculture, direct shoot regeneration etc., was transferred to 1/2 MS plus 44.4 μM BA and 0 μM 2,4-D (D6, 0.0).

d) Callus derived from various sources e.g., immature embryos, 1/2 MS, (D6, 0.0), NAA x BA, root tip subculture, was transferred to 1/2 MS.

e) Callus derived from 1/2 MS, NAA x BA, and immature embryos was transferred to (a) 1/2 MS plus 4.54 μM TDZ and 4.14 μM picloram (M1,1), (b) 1/2 MS plus 4.54 μM TDZ and 2.07 μM picloram (M1,0.5), and (c) 4.54 μM TDZ (M1).

f) Immature embryos, callus from (M1, 0.5), and callus from (M1,1), were transferred to 1/2 MS plus 90 g L^{-1} sucrose, 10% (v/v) coconut water, and 10 μM 2,4-D. To counteract bacterial contamination, filter sterilised kanamycin sulphate (Boehringer Mannheim GmbH) was incorporated at 50 μg ml^{-1} into some of the media in the final stages of this trial.

2.2.2.2 Shoot organogenesis induction trials:Subcultures

a) Subculturing of callus, derived from the factorial plant growth regulator trial (2.2.1 c), on media of the same composition that the callus derived from, was undertaken to investigate subculturing responses and also attempt to induce organogenesis (designated subculture round I). Large clumps (10-15 mm diam.) of callus were cut into small (3-5 mm diam.) pieces and placed firmly in contact with the medium.

b) Subculture round II involved subculturing callus from various plant growth regulator concentrations onto media of the same composition as that from which the callus derived and included callus from subculture round I.

c) In subculture round III, all material was derived from subculture round II and was plated out on media of the same composition that induced the callus.

d) Root tip subculture involved removing the root tips from *C. palmensis* roots that arose on 1/2 MS plus 0 μM BA and 2.26 μM 2,4-D and plating them on media of the same composition. Root tip sections 5-10 mm in length were used.

2.2.2.3 Shoot organogenesis induction trials:Induction

a) Direct shoot regeneration was attempted on 1/2 MS plus 22.2 μM BA using surface sterilised cotyledons, derived from 5 day-old aseptically germinated seedlings, as explant material. Cotyledons were bisected longitudinally and placed flat on the medium. In this experiment, changes of callus fresh weight, over time, were recorded.

b) Using surface sterilised cotyledons, callus induction was initiated on 1/2 MS plus 8.88 μM BA and 2.26 μM 2,4-D.

c) Surface sterilised cotyledons were used to induce callus on 1/2 MS plus 22.2 μM BA and 9.04 μM 2,4-D.

d) Using surface sterilised immature seeds a factorial plant growth regulator trial was conducted on 1/2 MS plus BA at concentrations of 0, 2.22, 4.44, 8.88, 22.2, or 44.4 μM and 2,4-D at concentrations of 0, 2.26, 4.52, 9.04, 22.32, or 45.24 μM. Immature seeds were aseptically removed from surface sterilised (2.5% Cl, 10 minutes) pods and then entire immature seeds were placed in contact with the medium.

e) Surface sterilised immature cotyledonary nodes, derived from immature seeds, were used as the explant material for callus induction in a semi-factorial trial conducted on 1/2 MS plus BA at concentrations of 0, 11.1, or 22.2 μM and NAA at concentrations of 0, 13.4, or 26.9 μM. The cotyledonary node is what

remains when most of the immature cotyledons and nearly all of the immature embryo is removed (Grant *et al.*, 1995).

f) Surface sterilised apical and sub-apical shoot tips derived from *in vitro* sources were cultured on (a) 1/2 MS plus 4.54 μM TDZ and 2.07 μM picloram (M1, 0.5), and (b) 1/2 MS plus 4.54 μM TDZ and 4.14 μM picloram (M1,1). Shoot tips were excised and attached leaves were reduced in size until the explant was approximately 5 mm in diameter. Explants were placed vertically in medium with apex uppermost. PPM was used at 1.0 ml L^{-1} in attempts to overcome contamination.

g) Cotyledons, hypocotyls, and roots, from aseptically germinated seed were aseptically surface sterilised (2.5 % Cl + Tween 20 for 15 minutes, followed by 3 x dH_2O rinse). Meristems were removed, explants were bisected and placed on media (A), (B), (C), and (D). After 21-28 days callus from these treatments was transferred to media C2.0, (C), (D), C3.3, M1.

2.2.3 Shoot multiplication *in vitro*

Initially, shoots were transferred from the medium they were grown on, to a medium of the same composition. Shoot tips were aseptically excised at approximately 20-30 mm from the apex, leaving 2-3 nodes on the shoot, which was then placed vertically, with the base inserted approximately 10 mm into the fresh medium. Each 250 ml container held 4-6 shoots. All shoot cultures were kept in a growth room with continuous light (approximately 50 μmol m^{-2} s^{-1}) at 22° C. After approximately 4 months, routine shoot multiplication was conducted on 1/2 MS only, supplemented with PPM at 1.0 ml L^{-1} to control contamination as required. When the majority of cultures became axenic, the use of PPM was discontinued. *In vitro* shoots were subcultured approx. every 3 months.

2.2.4 Propagation of *C. palmensis* clones

Shoots that rooted spontaneously *in vitro* were removed from the tissue culture media non-aseptically, washed thoroughly with running water to remove agar from their roots, and were transferred to a mix of sharp sand, perlite, and peat in a ratio of 2:2:1 in small plastic pots. They were then moved to an *ex vitro*

environment (16 h daylight at 22° C), fertilised with 25 mg of Phostrogen (Phostrogen Ltd., Great Britain), and covered with pre-cut 1.5 L plastic soft drink bottles to maintain high humidity. Fungal pathogens were controlled with Thiram at 1.5 g L^{-1}. After two to four weeks, rooted clones were moved to standard glasshouse conditions. Attempts were made to induce roots on some of the *C. palmensis* clonal shoots that had a 'wet' appearance *in vitro*. (see results section). Excised shoot tips, 20-30 mm in length, were placed vertically on 1/2 MS plus 4.92 μM IBA with their bases inserted approximately 10 mm into the medium. This was carried out on clones 1, 2, 4, and 5.

2.3 Change of *C. palmensis* callus fresh weight

The callus formed on surface sterilised cotyledons was subcultured on 1/2 MS plus 22.2 μM BA. The fresh weight of the callus was determined aseptically, as accurately as possible, on a four decimal place balance (AND ER 182-A) at 7, 14, 21, and 28 days from the start of subculture. This experiment was repeated 3 times. Callus was carefully removed from a culture jar, aseptically, and placed in a pre-weighed sterile Petri dish, which was then weighed. After weighing, the callus was returned aseptically to its original culture medium.

(3) LEAD EFFECTS

3.1 *P. radiata* seedlings in lead nitrate solution

Surface sterilised stratified seeds were transferred to sterile moist vermiculite, for germination at 26° C in the dark. Initially, twelve uniform seedlings, with cotyledons just beginning to expand, were selected. Six were exposed to 1208 μM $Pb(NO_3)_2$ and six were placed in dH_2O as controls, all in McCartney bottles containing approximately 10-15 mls of solution topped up as required, in the growth room. No nutrients were supplied. Observations were

carried out daily, on gross morphological changes including shoot formation, root colour, and general vigour. After 16 days in solution, one treated seedling and one control seedling were selected and sent to the SEM for analysis (appendix I).

In a subsequent trial, twenty five uniform seedlings with cotyledons just beginning to expand, were selected and five each were transferred to $Pb(NO_3)_2$ at 0, 151, 302, 604, and 1208 μM in McCartney bottles (10-15 mls) topped up as required. These were arranged randomly in the growth room. No nutrients were supplied. Observations were carried out approximately every two to three days for signs of stress, colour changes, growth inhibition and collapse. This experiment was concluded after approximately 7 weeks. A second round was conducted under identical conditions for approximately 8 weeks, when photographs were taken of some of the experimental units.

3.2 Seedlings in lead nitrate solution: Fresh weight changes

Twelve uniform seedlings of either *P. radiata* or *C. palmensis* were selected that had been growing in HC nutrient (appendix B) for 12 days since germination. They were washed with dH_2O, blotted dry on paper towels, weighed, and placed in each of the four different levels of $Pb(NO_3)_2$ (0, 20, 50, and 100 μM) in the HC nutrient solution, at pH 4.5, in McCartney bottles. The vials contained 15-20 ml solution, were arranged completely randomly, in the growth room. At day 14, the experiment was terminated, the length of the longest secondary root as well as the fresh weight of 3 seedlings in each treatment were recorded. This experiment was repeated three times each for both *P. radiata* and *C. palmensis*.

3.3 *In vitro P. radiata* shoots grown in a medium supplemented with lead nitrate

Clonal *in vitro* shoots were aseptically transferred to deep 90 mm plastic Petri dishes containing 20 ml full strength LPO medium plus $Pb(NO_3)_2$ at

concentrations of 0, 20, 50, 100, and 200 μM . The pH of the medium was adjusted to between 5.6 and 5.8 prior to autoclaving. Minor cloudiness due to co-precipitation of Pb^{2+} and PO_4^{3-} (from the LPO) was evident in the 200 μM solution and to a lesser degree in the 100 μM solution. All other solutions were clear (subsequently, precipitation was reduced by using half-strength LPO). Each plate contained three healthy, uniform shoots from the same clone and all needles that were brown, damaged or discoloured were removed prior to transfer. Observations were made weekly on colour, needle tip condition, and general vigour. After seven weeks, needles free of agar were weighed. The experiment was conducted seven times, using clones 2, 4, 5, and 9.

3.4 Effect of lead nitrate on seed germination

3.4.1 *P. radiata*

(a) Surface sterilised seeds, stratified for 14 days in dH_2O, were germinated in the dark at 26° C, in Petri dishes containing $Pb(NO_3)_2$ in dH_2O at concentrations of 0, 20, 50, 100, 200, and 1000 μM. Each plate contained 10 ml of solution and 8 seeds. In addition, one plate contained 10 ml 100 μM $NaNO_3$ and 8 seeds to provide a positive control for NO_3^- in the absence of Pb. After 14 days the experiment was concluded and the germination percentage was scored. This trial was conducted twice.

(b) In a four-way trial, where all seeds were surface sterilised;

1. ten seeds were stratified for 14 days in 10 ml dH_2O at 26° C in the dark, before they were moved to a growth room with continuous light.
2. ten seeds were stratified for 14 days in 10 ml dH_2O at 26° C in the dark, before they were rinsed and transferred to 10 ml 1000μM $Pb(NO_3)_2$, after this, they were kept in the growth room with continuous light.
3. ten seeds were stratified for 14 days in 10 ml 1000μM $Pb(NO_3)_2$ at 26° C in the dark before they were moved to the growth room with continuous light.

4. ten seeds were stratified in 10 ml 1000μM $Pb(NO_3)_2$ at 26° C in the dark before they were rinsed and transferred to 10 ml dH_2O, after this, they were kept in the growth room with continuous light.

After 4 weeks, the trial was concluded and the germination percentages were scored. This trial was conducted twice.

3.4.2 Multiple species

Seeds of several species were germinated in 1000μM $Pb(NO_3)_2$ or dH_2O to determine their sensitivity to Pb. Usually ten seeds at a time from each species were placed on filter paper in either 90 mm or 50 mm (depending on seed size) plastic Petri dishes containing 10 ml or 5 ml of either dH_2O or $Pb(NO_3)_2$ and placed at 26° C, in the dark, for seven days. *P. abies* seeds were kept at 26° C, in the dark, for 14 days and *C. palmensis* seeds (30 per Petri dish) remained in the dark for between 22 and 26 days. Observations included germination percentage, root and shoot elongation, and root tip discolouration. The species chosen for this study included:

Monocots: corn (*Zea mays*) NK 51036, ryegrass (*Secale cereale*) 'Ruanui', wheat (*Triticum aestivum*) 'Otane'.

Dicots: radish (*Raphanus sativus*) 'Salad Crunch', white clover (*Trifolium repens*), lettuce (*Lactuca sativa*) 'Great Lakes', and tree lucerne (*C. palmensis*).

Gymnosperm: Norway spruce (*Picea abies*).

C. palmensis seeds were treated as before (section 1.2) to break seed coat-imposed dormancy. Wheat, corn, clover, and *C. palmensis* seeds were all surface sterilised for 15 minutes in 2.5 % available Cl in dH_2O prior to germination. Some lettuce seeds that had been subjected to 1000 μM $Pb(NO_3)_2$ for seven days were transferred to dH_2O for a further seven days for a recovery trial. Photographs were taken of the germination response of the lettuce seeds to 1000 μM $Pb(NO_3)_2$.

(4) LEAD UPTAKE STUDIES

4.1 Flame atomic absorption spectrometry (a.a.)

All quantitative Pb analysis was conducted using a Varian AA-1475 series flame atomic absorption spectrometer. The machine was calibrated each time it was used with analytical grade 1000 ppm $Pb(NO_3)_2$ (BDH) diluted to 5, 10, 15, and 20 ppm in 1% HNO_3 (BDH). The machine was zeroed with 1% HNO_3, and three absorbance readings at either 217 or 283 nm were taken at each level, using a Pb hollow cathode lamp, and a calibration curve was constructed from the mean absorbance values (appendix K). The slit gap was set to 1.0, current to 5 mA, oxidant level to approx. 30-35, and the fuel (acetylene) set to approx. 8-10. When required, the machine was re-calibrated with either 50, 100, 150, 200 ppm $Pb(NO_3)_2$, or 200, 400, 600, 800 ppm $Pb(NO_3)_2$, depending on the Pb concentrations in the plant materials. In all trials, for all samples, three absorbance readings were taken at each level and the arithmetic means of these were interpolated against calibration curves to provide Pb uptake levels in ppm.

4.1.1 Conversion from ppm to mg kg^{-1}

All plant samples subjected to flame a.a analysis for Pb content weighed 1 g (d.w.) or equivalent and were dissolved in 10 ml of 2 % (v/v) HNO_3. Readings obtained in parts per million (ppm) are equivalent to mg L^{-1} (1 ppm = 1 mg L^{-1}). At 1000 ppm, 1000 ml of a solution contains 1000 mg (or 1 g), of a substance e.g., Pb, and 10 ml of the same solution contains 10 mg. At this concentration, therefore, a 1 g (d.w.) plant sample dissolved in 10 ml contains 10 mg of Pb and, by extrapolation, a 1000 g (d.w.) plant sample contains 10,000 mg (or 10 g) of Pb.

Simply put, multiplying ppm readings by 10 converts them to mg kg^{-1}, e.g., 1000 ppm multiplied by 10 equals 10,000 mg kg^{-1} (or 10 g kg^{-1}). This represents dry mass accumulation of 1% (w/w).

4.2 Sample preparation for flame a.a

4.2.1 Calculation of fresh weight:dry weight ratio

The most convenient sample size for flame a.a. analysis was deemed to be 1 g dry weight before ashing, due to the size of the silica crucibles and for plant Pb uptake calculations. To determine the optimum plant size for uptake experiments for both *P. radiata* and *C. palmensis*, the ratio of fresh weight to dry weight was determined. Seedlings of both species were collected, separated into roots, shoots, and whole seedlings, dried in a 60° C oven (desiccated), and weighed at 24 h and again at 28 h, to ensure a constant dry weight determination.

4.2.2 Dry ashing of plant material

After Pb exposure, the roots of the intact seedlings/clones were routinely desorbed (Huang and Cunningham, 1996) for 30 min in either 1.0 mM H-EDTA, (Sigma) or 1.0 mM EDTA-Na_2, (BDH), in HC nutrient at pH 4.5 to remove apoplastic Pb, rinsed three times in dH_2O, and blotted dry on paper towels. Roots and shoots were separated and desiccated overnight in a 60° C oven. Silica crucibles were previously acid washed for 24 h in 10% HNO_3 (v/v), rinsed three times in dH_2O, and dried at room temperature. One gram of dried plant material was placed into each crucible and ashed at 550° C for approximately 18 h. After cooling, 10 ml of 2% HNO_3 (v/v) were added to the ash in each crucible, mixed thoroughly, and transferred quantitatively to labelled McCartney bottles.

4.3 Plant Pb uptake and analysis

4.3.1 Vacuum filtration in lead nitrate solution

Needles from *P. radiata* clones T and U were vacuum filtrated in 50 ml of 50 μM and 100 μM $Pb(NO_3)_2$ for approximately 3 h. Treated needles were desorbed in 1.0 mM H-EDTA in HC nutrient at pH 4.5 for 30 min. Samples were desiccated at 60° C overnight, 0.5 g of each ashed at 550 ° C for approximately 18 h., and analysed by flame a.a.

4.3.2 Pb uptake in soil-less media

Uniform 6 month-old *P. radiata* (25-30 cm height) and *C. palmensis* seedlings (60-80 cm height) glasshouse-grown in 100 mm pots, in approximately 350 g (d.w.) soil-less media comprising sand, perlite and peat (2:2:1), were moved to 22° C, 16 h light. Initially, seedlings were watered to field capacity daily, with HC nutrient at pH 4.5, for several days. This was to remove as much P as possible to minimise co-precipitation of P and Pb during treatment, and to acclimatise the roots to a low pH (4.5) environment. Lowering the pH of the root zone also reduced Pb precipitation. Initially $Pb(NO_3)_2$ was added as a single application of 155 ml at the appropriate Pb concentration in HC nutrient at pH 4.5 (155 ml represents the quantity of retained moisture in soil-less medium in a full 100 mm pot at field capacity). Thereafter, daily watering was carried out with HC nutrient at pH 4.5 as required. In subsequent trials, $Pb(NO_3)_2$ was added at the appropriate concentration, in HC nutrient at pH 4.5, to field capacity initially, then daily as required. Plants were watered as necessary with HC nutrient and in all cases roots were desorbed with 1.0 mM H-EDTA in HC nutrient at pH 4.5, post-treatment. Samples of treated materials were desiccated, ashed, and analysed by flame a.a.

Treatments of *P. radiata* seedlings with different concentrations of lead nitrate and chelators:

- 0, 100, 200, 1000 μM, applied once as 155 ml, for 18 days.
- 0, 100, 200, 1000, 1500, 2000 μM, applied once as 155 ml, for 18 days.
- 0, 100, 200, 1000 μM, applied continuously, for 7 days.
- 0, 100, 200, 500 μM, applied continuously, plus 2g H-EDTA kg^{-1} mix (0.6 g per pot) applied once at each level, for 7 days.
- 0, 500, 500 (+ H-EDTA), 1000, 1000 (+H-EDTA) μM, applied continuously for 7 days. H-EDTA applied once at 0.5 g per pot.

Treatments of *C. palmensis* seedlings with different concentrations of lead nitrate:

- 0, 100, 200, 1000 μM, applied continuously, for seven days.

4.3.3 60 minute uptake

Initially, batches of very small, approximately 6 week-old uniform seedlings (10 cm total length) were pooled to provide enough biomass for flame a.a. analysis. These seedlings were grown in HC nutrient in 16 h light at 22° C. Later, larger 12 week-old uniform seedlings (15 cm total length) growing in sand culture in glasshouse conditions were included. In all cases, they were transferred to 20 μM $Pb(NO_3)_2$ in HC nutrient at pH 4.5, in continuous light at 22° C, for 60 minutes (in some cases, 1.44 mM H-EDTA was included in the trial and in one case gentle orbital shaking was included). After removal from solution, they were rinsed three times with dH_2O, desorbed for 30 minutes in 1.0 mM H-EDTA in HC nutrient at pH 4.5, desiccated overnight, dry ashed, and analysed by flame a.a.

4.3.4 Pb uptake in solution

Eventually uptake experiments were conducted solely in hydroponic solution, for seven days, with and without a chelator which was lowered from 1.44 mM, to 0.5 mM H-EDTA or EDTA, as plants showed symptoms of stress at the higher concentration (Vassil *et al.*, 1998). $Pb(NO_3)_2$ concentrations were either 500 μM or 250 μM in HC nutrient at pH 4.5. Chelator concentrations for *C. palmensis* seedling uptake trials were eventually lowered to 0.125 mM for both EDTA and H-EDTA and pH adjustments for all solutions were made with 1 M KOH, not NaOH, to reduce possible excess sodium effects. Initially, glasshouse grown 8-12 month-old *P. radiata* seedlings (25-70 cm) and *C. palmensis* seedlings (70-100 cm), were removed from sand culture and washed carefully to remove the soil-less medium from their roots to prevent damage to intact root surfaces. When necessary two or three seedlings per 500 ml container were used to obtain sufficient plant material for analysis. Containers were topped up as necessary with solutions of the original composition. Initially, solutions were not aerated but in all later uptake trials continuous aeration was in place to prevent growth of pathogens due to anaerobic conditions.

Uniform clonal materials of both species were included in the studies when they had attained sufficient size. *P. radiata* clones S, T, V, W, and X were

between 30 and 50 cm in height, had well developed root systems, vigorous top growth and mature-type needles. *C. palmensis* clones 1, 2, 5, and 7 were between 40 and 90 cm in height, had well developed root systems and vigorous top growth. Chelator concentrations for Pb uptake with *C. palmensis* clones were 0.125 mM H-EDTA and 0.5 mM EDTA. At the conclusion of each trial, plants were desorbed, desiccated overnight, dry ashed, and analysed by flame a.a. All desorption was conducted with 1.0 mM EDTA-Na_2 in HC nutrient at pH 4.5 for 30 minutes (Huang *et al.*, 1997). Plant samples for transmission electron microscopy included *P. radiata* and *C. palmensis* shoots and roots at 0 Pb, 500 µM Pb, 500 µM Pb + 0.5 mM H-EDTA, 500 µM Pb + 0.5 mM EDTA, and *C. palmensis* root nodules from the treatments at the same concentrations.

(5) TRANSMISSION ELECTRON MICROSCOPY

5.1 Preparation of ultra-thin sections

5.1.1 Fixation

Both Pb-exposed and non Pb-exposed root tips, root nodules, needles or leaves from both species were cut into smaller pieces by scalpel and placed in small glass vials in fixation buffer containing 3% glutaraldehyde (v/v) in 0.075 M sodium phosphate buffer, under partial vacuum, for 3 to 4 hours at room temperature. The buffer was changed twice, to 0.075 M phosphate, for 10 minutes each time, and then once more for 30 minutes. The specimens were then placed in OsO_4 in 0.075 M phosphate buffer, for 3 hours, followed by a buffer change, overnight.

5.1.2 Dehydration

The tissue was then dehydrated in small glass vials in an acetone series of 20%, 40%, 60%, and 80%, for 10 minutes at each step, followed by 100%, three times, each for 15 minutes.

5.1.3 Infiltration

In the first infiltration step the specimens were placed in small glass vials in 1/3 Spurrs (Electron Microscopy Scientific Ltd., U.S.A.) resin : 2/3 100% acetone on a slowly rotating wheel overnight. The second infiltration step utilised 3/4 Spurrs resin : 1/4 100% acetone, on the wheel for at least 3 hours.

5.1.4 Embedding

The specimens were embedded in 100% Spurrs resin, in shallow plastic caps, placed in a glass Petri dish in a 50° C oven, overnight.

5.1.5 Ultramicrotomy

Suitable specimens were cut from the resin blocks and glued with epoxy resin to 'stubs' (OO capsules filled almost to the top with hardened epoxy resin). These were allowed to set at room temperature, overnight. The stubs were trimmed to produce extremely small, flat, surfaces exposing the desired part of the specimen. Once trimmed the stubs were mounted in an ultramicrotome (LKB 212B Ultratome) and approximately 90-100 nm ultra-thin sections were prepared and mounted on copper TEM grids.

5.1.6 Staining of ultra-thin sections

Most sections viewed in the TEM were unstained as sufficient details were observed. However, some *P. radiata* sections were stained for comparative purposes. These sections, still on the copper grids, were placed in 1% uranyl acetate (v/v) in 50% ethanol for 10 minutes, followed by modified 3% (v/v) Reynold's reagent (Hayat, 1975) containing three types of Pb compounds (nitrate, acetate, and citrate) in sodium citrate for 5 minutes.

5.2 Microscopy

Ultra-thin sections were viewed in the transmission electron microscope (Jeol-1200 EX) at an accelerating voltage of 80.0 kV. Micrographs were taken of

all regions of interest on Kodak Estar electron microscopy sheet film (8.3 x 10.2 cm).

(6) ACID PHOSPHATASE

6.1 Qualitative assay

XP (5-bromo,4-chloro,3-indolyl-phosphate-ρ-toluidine) a dye-linked acid phosphatase substrate used in qualitative assays was applied to plant root tissue to visualise root surface-associated acid phosphatase, EC 3.1.3.2 (Rothe, 1994) activity. Exposure to acid phosphatase results in the cleavage of XP, producing a blue pigment (Goldstein *et al.*, 1989). The working concentration of XP (Sigma), was obtained by first dissolving 2 mg in 100 μL of dimethylformamide (DMF), (BDH) and then adding it to 25 ml of solution, either dH_2O or $Pb(NO_3)_2$. Initially *P. radiata* germinated seeds and 14 day-old seedlings, and *C. palmensis* 14 day-old seedlings were exposed to XP in dH_2O for 5 hours at 26° C in the dark. Subsequently seedlings of both species were exposed to XP added to 20 μM $Pb(NO_3)_2$ in dH_2O, for 18 hours, to determine if Pb in the solution interfered with the cleavage reaction.

6.2 Quantitative assay

Acid phosphatase activity was estimated by colourimetrically measuring the absorbance of the product formed by the cleavage of the artificial substrate ρ-nitrophenyl phosphate (Sigma), which was used at a working concentration of 50 mM. The prepared solution was kept on ice, wrapped in foil, until required, to prevent artefactual colour development.

6.2.1 Cell-free tissue extracts

Selected plant material, including Pb-exposed and non Pb-exposed roots and shoots of both species, was excised from intact plants, washed with dH_2O, and blotted dry with paper towels. In addition assays were conducted on tissue that had been in a zero P (dH_2O) treatment. Approximately 1.0 g (f.w.) of tissue was placed in a pre-chilled mortar on ice and ground to a fine powder in liquid N_2. In 30 ml centrifuge tubes, the powder was resuspended in 0.1 M citrate buffer (pH 5.0) (appendix D) at the rate of 50 mg tissue ml^{-1} buffer. For tissue with high phenolic levels, such as leaves, insoluble polyvinyl polypyrrolidone (PVPP, Sigma) was added to the citrate buffer at 1% (v/v) as well. These were kept on ice for 20 min. with sporadic vortexing after which they were centrifuged (Eppendorf 5403) at 11,000 rpm, at 2° C, for 10 minutes. The supernatant, or cell-free tissue extract, was carefully removed and the volume determined. To account for losses that occurred in preparation, usually a small amount of buffer was added to bring the volume to a round number to simplify calculations. The extract was then decanted into labelled 1.5 ml Eppendorf tubes and stored at either 4° C for a few days, or -20° C for longer periods.

6.2.2 Reaction mixtures and incubation

Reaction mixtures consisted of 0.25 ml 0.1 M citrate buffer (pH 5.0), 0.25 ml of 50 mM ρ-nitrophenyl phosphate in 0.1 M citrate buffer (reaction concentration of 16.67 mM), and 0.25 ml of crude enzyme extract, making a total of 0.75 ml. All reagents and extracts were kept on ice prior to the assay. Assays were conducted in a water bath at 37° C. All ingredients were added to test tubes, on ice, vortexed, then moved to the water bath for incubation. Control tubes contained only extract and buffer. For *P. radiata*, usually three different incubation times were used, e.g., 30, 60, and 90 minutes. For *C. palmensis*, usually assays were conducted for 5 minutes with three different extract volumes e.g., 0.05, 0.1, and 0.15 ml (the volume of the reaction mixture was maintained at 0.75 ml by substituting citrate buffer for crude extract) and when necessary, shoot extracts were diluted up to 1:6. After incubation, all test tubes were moved to ice and the reaction was stopped by the addition of 0.75 ml of cold 1 M NaOH. These

were vortexed and returned to ice. Substrate (0.25 ml) was added to control tubes and vortexed. After colour development, absorbances were measured at 410 nm in a Novaspec II spectrophotometer (Pharmacia Biotech). Details of conversion of absorbance values to units of enzyme activity g^{-1}(f.w.) min^{-1} are presented in appendix G.

6.3 Total soluble protein determination

To relate acid phosphatase activity to total soluble protein content, assays were conducted on selected batches of cell free tissue extract for both *P. radiata* and *C. palmensis* according to the method of Bradford (1976). Absorbances were measured at 595 nm in a Novaspec II spectrophotometer. The machine was blanked with a mixture of 100μL dH_2O and 1 ml of Bradford reagent. Subsequently, protein concentrations were determined by adding 100 μL of enzyme extract to 1 ml of Bradford reagent, vortexing, waiting approximately 5 minutes, then measuring absorbance. A standard curve against bovine serum albumin (BSA, BDH) had previously been obtained.

(7) ACID PHOSPHATASE ISOZYME GEL ELECTROPHORESIS

7.1 Apparatus

Native (non-denaturing) polyacrylamide gel electrophoresis (PAGE) was carried out, using the method of Laemmli (1970) with modifications, in a Studier-type slab gel apparatus (Hames, 1981). Power was supplied with an ES303 power supply (Solstat Industries Ltd.). Gels were cast between glass plates (150 x 160 with 1.5 mm spacers) sealed with 1% agar.

7.2 Reagents

Acrylamide and fast garnet GBC were supplied by BDH. Ammonium persulphate (APS), tetramethylenediamine (TEMED), and N,N'-methylene bisacrylamide (BIS) were supplied by Bio-Rad (Hercules, U.S.A.). Aminoaceticacid (glycine), β-alanine, (Tris[hydroxymethyl]aminomethane) (Tris), Tris-HCl, and β-naphthyl acid phosphate were obtained from Sigma (St. Louis, U.S.A).

7.3 Procedure

7.3.1 Sample preparation

Cell free tissue extracts were made from tissue samples of seedlings and clones (2,7) of *C. palmensis* and clones (T,U) of *P. radiata*, some of which had been exposed to $Pb(NO_3)_2$, in the presence or absence of a chelator. Pb-exposed material was desorbed with 1.0 mM EDTA in HC nutrient at pH 4.5 for 30 minutes. Extracts were either used fresh or stored at -20° C. Prior to loading onto gels, all extracts were concentrated either by dialysis in 20 kD polyethylene glycol (PEG, BDH) (at room temperature or 4° C), or acetone (BDH) precipitation.

Dialysis was carried out by placing approximately 1-3 ml of extract into a pre-wetted 100mm section of dialysis tubing, knotted twice at each end. This was placed into enough PEG to completely cover the dialysis tube and as the PEG absorbed moisture, it was scraped from the outside of the dialysis tube and replaced by fresh PEG. When the volume of extract had reduced to approximately 1/4-1/2 of the original volume, which typically took 1-2 hours, the dialysis tube was removed from the PEG, rinsed in dH_2O, and the concentrated extract was either stored overnight at 4° C, or prepared for loading on the gel.

Acetone precipitation required diluting cell-free tissue extracts 1:4 with ice-cold (-20° C) acetone. Usually 300 μL of extract were added to 1200 μL acetone, in a 1.5 ml Eppendorf tube, and stored at -20° C overnight (immediately after adding the acetone, particularly with *C. palmensis*, a dark, rubbery

precipitate formed that was easily removed with a 20 μL pipette). Acetone/extract mixtures were then centrifuged at 12,000 rpm for 10 minutes after which the acetone was carefully poured off. Any remaining acetone was removed by vacuum evaporation for approximately 60 seconds. The pellet was resuspended in minimal 0.1 M citrate buffer (pH 5.0), vortexed and/or pipetted until the pellet was dissolved, and spun again at 12,000 rpm for 10 minutes. After this, the supernatant was decanted and prepared for loading on the gel.

7.3.2 Gel preparation, loading, and running conditions

(a) **Native high pH gels**: Uniform 3% T stacking gels (30 x 140 x 1.5 mm) made with 0.126 M Tris-HCl (pH 6.8), and 8% and 12% T resolving gels (100 x 140 x 1.5 mm) made with 0.375 M Tris-HCl (pH 8.8) were prepared as per Hames (1990). Acrylamide solutions were de-gassed for at least 20 minutes prior to polymerisation with APS and TEMED. Resolving gels typically took approximately 1 hour to set, and stacking gels took approximately 20-30 minutes. The reservoir buffer consisted of 0.454% (w/v) Tris and 2.16% (w/v) glycine. The tracking dye was 0.003% (w/v) bromophenol blue. Before being loaded on the gels, both the extracts and the dye were diluted with 60% (w/v) sucrose to contain a final concentration of 20 % sucrose. Electrophoresis was carried out at 4° C, at 60-65 mA through the stacking gels, which took 15-20 minutes, after which the current was turned down to 25 mA until the tracking dye nearly reached the end of the resolving gels which took, on average, 4 hours. After removal from the apparatus, gels were equilibrated in 100 ml of 0.1 M citrate buffer (pH 5.0) at 4° C for 5-15 minutes.

(b) **Native low pH gels**: Uniform 3% T stacking gels (30 x 140 x 1.5 mm) made with 48% (v/v) 1M KOH plus 2.9% (v/v) acetic acid in dH_2O (pH 6.8), and 8% and 12% T resolving gels (100 x 140 x 1.5 mm) made with 48% (v/v) 1M KOH plus 17.2% (v/v) acetic acid in dH_2O (pH 4.3) were prepared as per Hames (1981). Acrylamide solutions were de-gassed for at least 20 minutes prior to polymerisation with APS and TEMED. Resolving gels typically took approximately 1-2 hours to set, and stacking gels took 30-60 minutes. The reservoir buffer (pH 4.5) consisted of 3.12% (w/v) β-alanine and 0.8% (v/v) glacial acetic

acid (BDH). The tracking dye was 0.1% (w/v) methylene blue. Before the extracts and the dye were loaded on the gel, they were diluted with 60% (w/v) sucrose to contain a final concentration of 20 % sucrose. Electrophoresis was carried out with reverse polarity, at 4° C, at 65 mA through the stacking gel (which typically took 25-30 minutes), and initially 25 mA through the resolving gel but this was later raised to 40 mA otherwise gels took 6-7 hours to run. After removal from the apparatus, gels were equilibrated in 0.1 M citrate buffer (pH 5.0) at 4° C for 5-15 minutes.

7.3.3 Gel staining for phosphatase activity detection

Following equilibration, gels were incubated with 100 ml of solution containing 1.93 mM $MgCl_2$ (May & Baker, England), 0.1 % (w/v) fast garnet GBC, and 0.271 mM β-naphthyl acid phosphate in 0.1 M citrate buffer (pH 5.0) at 37° C, in the dark. This enzyme assay solution was made fresh each time immediately prior to use. Gels were stained for 1.5-3 hours if sufficient band colour developed, otherwise they were incubated overnight. When colour development was satisfactory, i.e. appearance of the bands of enzyme activity, the gels were rinsed in dH_2O and photographed with conventional 35 mm colour slide film.

(8) DATA ANALYSIS (STATISTICAL METHODS)

8.1 Pb uptake data analysis

Model I anova was performed on data derived from all Pb uptake experiments where the experiment was repeated twice or more, either on seedlings or clones, and the measured values substantially exceeded the lower limits of detection. Prior to anova, all uptake data were log 10 transformed to ensure homogeneity of variances. Two-factor analyses of variance, without replication, were performed on (a) data for root Pb uptake, (b) data for shoot Pb uptake, and (c) the log 10 transformed ratio of root Pb uptake:shoot Pb uptake. In all cases factor 1 was 'treatment'. Where experiments were repeated as multiple rounds, the

factor 'round' was included as a block effect (factor 2). When different clones were analysed, the factor 'clone' was included as a block effect (factor 2). Full anova tables for Pb uptake data are presented in appendix E.

8.2 Quantitative phosphatase enzyme assay data analysis

Model I anova was performed on data derived from all quantitative enzyme assays where the experiment was performed more than once with seedlings or a single clone, or with different clones, involving Pb exposure, with and without chelation. Prior to anova, all data were tested to ensure homogeneity of variances. Two-factor analyses of variance, without replication, were performed on (a) data for shoot enzyme activity and (b) data for root enzyme activity. In all cases factor 1 was 'treatment'. When different clones were analysed, the factor 'clone' was included as a block effect (factor 2).

8.3 Unplanned multiple comparisons of means (Tukey test)

Unplanned multiple comparisons of means were carried out using the Tukey test with standard error for equal sample sizes on all data where analyses of variance found the effects due to treatment (factor 1) or clone (factor 2) to be significant. All compared means were ranked in order of magnitude and those that were not significantly different from each other are joined by a horizontal line. Unplanned multiple comparisons of means of Pb uptake data are presented in appendix F.

CHAPTER III

RESULTS

(1) PLANT PRODUCTION

1.1 Seed germination

Initially *P. radiata* seeds were stratified in dH_2O for only 7 days and this led to germination percentages typically of about 50%. When the length of this period was extended to 14 days, germination rates increased to greater than 90% in all cases and often 100%, 6-8 days after stratification.

The single most important factor in mature *C. palmensis* seed germination was boiling for 60 seconds. Other factors were important in increasing the germination rate but unboiled seeds had still not imbibed after 4 weeks on moist filter paper at 26° C in the dark. Initial germination attempts including boiling for 60 seconds and simple surface sterilisation led to germination rates of 40-50%. Later, when Tween 20 was added to the bleach solution, surface sterilisation conducted in sterile laminar flow, and all excess moisture drained from the plates after imbibition, germination rates increased to 75% on average, after 14 days, and occasionally as high as 90%.

1.1.1 Immature *C. palmensis* seed germination

Due to initial difficulties in achieving reproducible axenic seed germination, embryo rescue was attempted as immature seed pods are relatively sterile compared to mature seed. Mature *C. palmensis* seeds are jet black, small (4-5 mm length including aril), extremely hard, with a matt surface and contain embryos which are predominantly yellow in colour and gradually turn green during germination. Immature seeds that were large (7 mm average length including aril), bright green, and soft, had their embryos, which were predominantly green with 5 mm cotyledons, removed and placed in continuous light at 22° C. Some of the embryos were placed on moist filter paper, others were

partially desiccated, during which time their volume decreased by approximately 1/3. When the desiccated embryos were moistened with dH_2O they regained their original volume. All the embryos gradually lost colour over 14 days and none of them germinated. Subsequent trials with more mature embryos (highly immature seeds are very shiny and as they mature they decrease in size and become less shiny) yielded similar results. Embryo rescue was not achieved with any immature seeds or embryos.

1.2 Vegetative propagation of *P. radiata* clones

Vegetative propagation of shoot cuttings was much more successful than propagation of fascicles. Rooting percentages of cuttings under mist after approximately 11 months were typically 65-75% and in some cases 100% whereas rooting of fascicles under mist was often zero and never exceeded 10%. Fascicle propagation was discontinued when it became apparent that shoot propagation was faster and more efficient. Some of the clones rooted more readily than others with the order of the most amenable to the least being S, T, U, W, Z, Q, V, X, R, Y. In some cases root formation was quite extensive with tap roots up to 20 cm in length being produced. Whenever this happened, these long tap roots were shortened to approximately 5 cm to encourage a fibrous root system to develop.

(2) TISSUE CULTURE

2.1 Cotyledon culture of *P. radiata*

In producing meristematic tissue from *P. radiata* cotyledons, a window of opportunity existed during germination when the radicle had protruded to a certain length, approximately 0.5-2.0 cm. If the embryo was excised too soon it was difficult to separate the individual cotyledons and if it was excised too late the cotyledons did not form meristematic tissue as readily. It was also important to remove the tips of the cotyledons otherwise they elongated instead of producing

meristematic tissue. All cotyledon sections derived from each seed were placed together on LP5 in one Petri dish (Plate 1A), this was the basis of each clonal line.

Typically cotyledons curled up when first placed on the medium. Some, lying flat, remained in close contact with the medium but others curled away from the surface and these had to be aseptically reattached. Fungal contamination of plates containing non-surface-sterilised cotyledons was quite common and although typically not all cotyledons on any single plate were contaminated, in practice the plate was discarded as dis-infestation of existing plates was more time consuming than initiating new ones.

Initially the cotyledons increased in volume by swelling radially and during the next 2-4 weeks small nodular protuberances appeared on the previously smooth surface indicating incipient shoot formation. Transfer to LPO in 90 mm Petri dishes accelerated the formation of needles and after 6-7 weeks they were transferred to LPO in 250 ml tissue culture containers as the depth of the Petri dish (12 mm) was beginning to restrict their growth (Plate 1B). Originally 12 different clonal lines were initiated in this way. On LPO medium single shoots grew longitudinally and new shoots appeared laterally at the base of existing shoots. When subculturing, long shoots were shortened to 20 mm sections, and side shoots were separated from clumps, with 4-6 shoots per 250 ml jar being the optimum spacing. As some clonal lines produced copious needle growth, literally filling up the jars, at periodic intervals some of these shoots were removed from the containers aseptically and 'pruned' to reduce their volume before being returned to the medium (Plate 1C).

Responses of the individual clones to *in vitro* culture varied considerably. Some of the clones readily formed meristematic tissue from cotyledons and initially formed needles on LPO quite satisfactorily but when they were subcultured they began to lose vigour from the first subculture round, showing decreased growth rates and increasingly chlorotic foliage. Several lines declined rapidly and died and only four were left after 24 weeks. Of those four, two were clearly more vigorous with bright green foliage, abundant growth, and profuse shoot initiation. Subsequently, two additional vigorous clonal lines were

established *in vitro.* The final six *in vitro* clonal lines utilised were designated #1, #2, #3, #4, #5, and #9. Differences among the clonal lines included :

a) needle shape- some had very straight, coarse needles whereas others had fine, curly needles.
b) height- some grew much taller than others.
c) lateral shoot formation- some produced considerably more shoots than others.
d) growth rate- some clones grew more quickly than others.

2.1.1 Long-term subculturable meristematic tissue

In assessing the potential of *P. radiata* meristematic tissue for *in vitro* selection/screening, it was necessary to determine how well the cultures survived *in vitro* over time. The longest surviving line of meristematic tissue originally came from cotyledons that were surface sterilised in 100 % ethanol and plated out on LP5. After 21 days, meristematic tissue was forming and the explants were transferred to 1/2 LP 2.5. After 14 days on this medium both needle and meristematic tissue growth were evident. At day 35 the needles were partially elongating, the meristematic tissue was still viable and callus was appearing. These cultures remained on this medium for a further 6 weeks at which time green meristematic portions were subcultured to fresh 1/2 LP 2.5. After 5 weeks on this medium the meristematic cultures were still green and viable but not completely pathogen-free so the healthiest parts were subcultured to fresh 1/2 LP 2.5 containing PPM. After 6 weeks on this medium all the clumps were green, meristematic and healthy, with no needle elongation and they were subcultured to 1/2 LP 2.5. After 3 weeks they were still meristematic but not completely pathogen-free so they were finally subcultured to 1/2 LP 2.5 plus PPM where they survived for a further 6 weeks, eventually becoming non-viable.

These cultures survived for approximately 9 months in total, undergoing 5 subcultures during this time. The two next best performing lines lasted through 3 subcultures, for approximately 5 months in total before losing viability.

2.1.2 *P. radiata* clonal root induction

On water agar only, with or without sucrose, shoots did not develop callus and no root formation occurred after 28 days. When the preconditioning treatment (RIM) including IBA and NAA for 10 days was introduced, callus tissue had formed when the shoots were transferred to REM. On this medium, most shoots formed significant amounts of spongy callus, which continued to enlarge for up to 10 weeks, but very few (approximately 10 %) formed roots. These roots appeared to form from callus tissue. Shoots on RIM containing 9.84 μM IBA and no NAA, for 13 days, produced neither callus nor roots. When RIM was subsequently modified to include only 49.2 μM IBA for 12-13 days, most shoots produced ample callus tissue but very few (approximately 20 %) formed roots, mostly from callus, but some roots formed directly from the stem on clone #4 after 21-28 days (13 d on RIM, 8-15 d on REM). With RIM at 73.8-98.4 μM IBA for 15 days, callus growth was vigorous but no root formation occurred.

Many shoots formed large callus masses but no roots on RIM with 49.2 μM IBA for 14 days and usually there was a lot of top growth associated with these. In some cases, the majority of the callus was aseptically removed and transferred to fresh RIM containing 4.92 μM IBA for 11-13 days. Rooting percentages did not increase as a result but contamination rates increased due to the extra handling. It was observed that once roots formed in agar-based media they did not perform well. They tended to become darker, their growth rate slowed and they became very static in many cases. In an attempt to overcome this, a free-draining soil-less mix was used to replace REM, with RIM still containing 2.46 or 4.92 μM IBA for 14 days. Rooting percentages remained very low, less than 5%, and it was difficult to simultaneously maintain the humidity level while controlling fungal pathogens. The few rooted shoots from any treatment that survived the transfer to soil-less medium, hardening off, and relocation to the glasshouse, formed sturdy, vigorous plants. Approximately 70 % of shoots that formed roots did not survive this process. Incidentally, some clones (approximately 1 %) growing aseptically on LPO, in continuous light at 22° C, formed roots up to 30 mm in length spontaneously, but none of them survived the transfer to *ex vitro* conditions (Plate 1D).

2.2 *C. palmensis* callus induction

All early attempts at culturing *C. palmensis* on 1/2 MS used explants derived from surface-sterilised seed, germinated aseptically. From the outset, a red pigmented micro-organism began to infect most culture plates. This organism was examined by light microscope and found to be a rod-shaped bacterium. The bacteria were always found on the plates in close association with the explant, particularly where there was free water, a situation that frequently arose when explants were pressed firmly onto the medium. Often, surface sterilisation of seed did not eradicate the pathogen, possibly due to the shape and texture of the aril (Plate 2A). To overcome this, initially whole surface-sterilised seedlings (100% ethanol for 5 seconds) were aseptically placed on culture media. In some cases, this eradicated the bacterium and did not adversely affect the explant although leaves were affected more than stems. Often when only a section of a plate became contaminated, it was possible to subculture uninfected material to fresh media.

2.2.1 Cotyledons, hypocotyls & roots

C. palmensis explants on 1/2 MS (+/- 10 μM 2,4-D) displayed a variety of responses to *in vitro* culture. When whole juvenile seedlings were used, the rapid development of meristematic and callus tissue, particularly with 2,4-D, often obscured the origins of the de-differentiated cells so it was decided to culture surface-sterilised cotyledons, hypocotyls, and roots on separate plates. On 1/2 MS, cultured cotyledons sometimes remained differentiated, characterised by an abundance of trichomes, but most produced some callus tissue. On 1/2 MS plus 10 μM 2,4-D, cell masses grew rapidly, often displaying dark red, bright emerald green, pure white or transparent cell pigmentation with very dark red, almost black exteriors which frequently split open to reveal masses of bright green cells inside. Cultured hypocotyls developed green tissue on the surface along their axes, and white and pink spherical callus cells proliferated at the cut ends of the sections. Regions of the epidermis had dark red pigmentation.

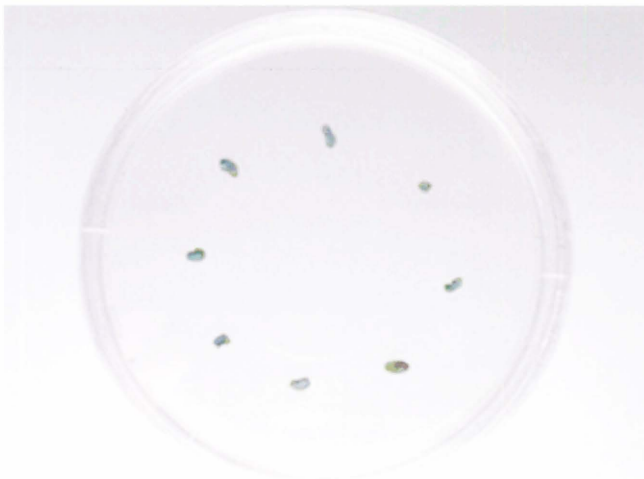
PLATE 1 Various stages of i*n vitro* culture of *Pinus radiata.*

(A) *Pinus radiata* cotyledons one week after transfer to LP5 medium.

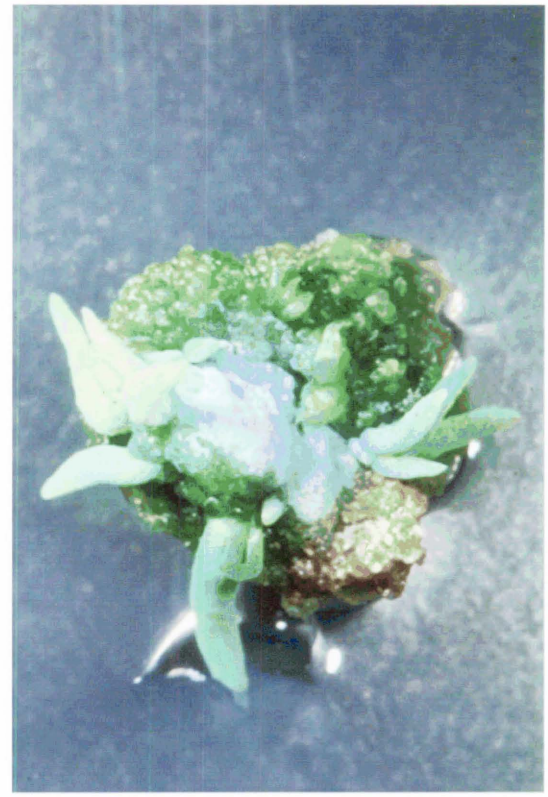
(B) *Pinus radiata* cotyledon after six weeks on LPO medium.

(C) *Pinus radiata in vitro* shoot after eight weeks on LPO medium.

(D) *Pinus radiata in vitro* root formation after four months on LPO medium.

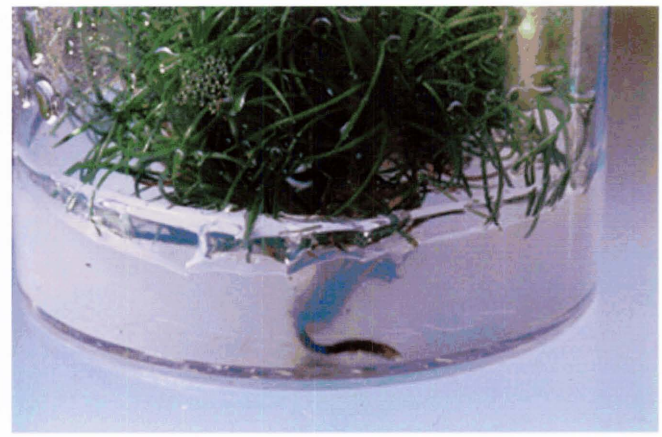


(**A**) Excised *Pinus radiata* cotyledons *in vitro* on LP5 medium.



(**B**) Needles forming from *Pinus radiata* meristematic tissue on LPO medium.

(C) *In vitro* shoot tip culture of *Pinus radiata* on LPO medium.



(**D**) Roots forming *in vitro* on *Pinus radiata* shoots on LPO medium.

PLATE 1

Whole roots, excised just below the hypocotyl produced creamy-brown coloured cells characterised by root hairs, and swollen regions comprised of green cells overlaid by parallel rows of well-organised red epidermal cells. In some cases these regions were disrupted by lumpy protuberances containing cells with a disorganised callus-like appearance. On average, 1/2 MS alone tended to produce slower growing, brown, callus-like cell growth whereas 1/2 MS plus 10 μM 2,4-D produced fast growing, green and multi-coloured cell masses.

2.2.2 Immature embryos

Whole immature embryos on 1/2 MS plus 10 μM 2,4-D were initially entirely green. After 10 days they were producing abundant callus/meristematic tissue and were growing rapidly. The cotyledons and associated cell mass were still green and meristematic but the radicle sections were a pale pink or brown and looked more like callus. After 5-10 weeks tiny, green, leaf-like structures began to appear amongst the meristematic cells. These structures were, at the very least, more differentiated than the cells surrounding them. When these structures were separated from the meristematic tissue and aseptically cultured on 1/2 MS, shoots formed. Part of the *in vitro* clonal collection was established by subculturing these shoots.

2.2.3 Factorial plant growth regulator trial I (BA & 2,4-D)

In selecting material for factorial plant growth regulator trial I, every effort was made to select uniform embryos but there was still considerable variation among them, particularly in colour. Embryo colouration included pale creamy yellow, bright green, green with patchy red pigmentation and almost white. Interestingly, some seeds contained two complete embryos.

Each replicate consisted of one Petri dish containing culture medium and six explants (the two cotyledons and one shoot apex from one seed, all bisected longitudinally). The intention was to place all explants in the trial onto media 9-10 days after initiation of germination, but in practice it was found that not all embryos developed uniformly with respect to time. Selection based on physical appearance as well as time was important as some embryos were quite green when

they were excised whereas others were very pale, almost white. The response of the pale embryos to the medium was often slower and in some cases they never turned green. Usually these explants remained very static on the medium and showed no response at all. It was found that selecting uniform yellow embryos with a little green colouration from the outset was more satisfactory.

As contamination from the red bacterium was still evident sporadically, replacement replicates were initiated when necessary. Although callus induction responses of explants to media composition ranged from modest to prolific, callus development was achieved at every level of the trial. Good callus induction was achieved after 7 days at some concentrations. Callus types produced included friable, non-friable, smooth, rough, lumpy, glossy, and matt. Colouration included green, brown, yellow, red, and white. Yellowish-brown to brown was the most common colour of the callus produced and was often very fast growing, with a high water content. Green callus was usually drier and more friable, red callus typically comprised spherical cells and white callus was often less dense and quite filamentous.

Some combinations were more successful than others, e.g., 2,4-D alone (at all levels) without BA produced mainly expansion of the explant after 35 days but minimal callus was obtained, even on 1/2 MS alone, whereas 22.2 μM BA x 2,4-D (at most levels) produced abundant callus. Possibly higher [BA] x lower [2,4-D] is slightly more effective at inducing callus. However, there is no definite pattern to callus induction in relation to the combination of growth regulators (Figure 1). During the course of the trial, each level of treatment was replicated twice and shoot induction occurred in approximately 12 of the replicates, presumably due to the inadvertent inclusion of shoot apical meristems. These shoots were subcultured and formed the basis of the majority of *in vitro* clones in the *C. palmensis* collection. Root formation occurred in one of the replicates.

2.2.4 Organogenesis induction trials

The aim of the experiments performed in this section was to induce *de novo* shoot organogenesis. They fell broadly into three categories:

(1) Transfer of *in vitro* material to media of a different composition.

(2) Subculture of *in vitro* material on media of the same composition.

(3) Induction of *in vitro* material on media of varied composition.

2.2.4.1 Transfers

Transfer of callus tissue from auxin-containing media to cytokinin-containing media, over a 28 day period, did not induce organogenesis. In one replicate, roots formed briefly on callus derived from hypocotyls. In others, callus became darker in colour, sometimes taking on a reddish hue, and protuberances began to appear in previously smooth callus. In general, callus growth slowed, most replicates became very static and at the conclusion of the experiment, very little green callus remained.

Transfer of *in vitro* tissue, predominantly callus, which originated on media containing various concentrations of BA (cytokinin) and 2,4-D (auxin), to media containing cytokinin only, did not induce shoot organogenesis after 28-35 days. Some shoot development occurred, presumably from pre-existing meristems, and limited root and hair growth occurred. Smooth callus often became nodulated, green colouration decreased and many replicates became quite static. Some necrosis occurred and from time to time any necrotic tissue was removed aseptically. Callus clumps were inverted occasionally as cells in direct contact with the medium tended to become quite oxidised and lose vitality over time. Oxidised cells became much darker in colour, sometimes almost black, and the ventral surfaces of callus clumps often became very hard and grainy. Transfer of callus to culture media incorporating TDZ and picloram produced several types of callus including white friable, transparent, hard green, and soft watery green. Some of the callus types were quite prolific in their growth patterns, literally filling up the Petri dishes. Callus produced on M1 medium (4.54 μM TDZ) was very grainy and nodulated whereas M1, 1 (4.54 μM TDZ & 4.14 μM picloram) and M1, 0.5 (4.54 μM TDZ & 2.07 μM picloram) generally produced a smoother, paler green callus (Plate 2B).

Of the three callus origins, (1)1/2 MS, (2) NAA x BA, and (3) immature embryos, the callus from the embryos performed the best but shoot organogenesis was not achieved after 6-7 weeks. The fastest growing forms of callus tissue

produced on these media were more resistant to oxidisation of the ventral surface, retained their colour, remained relatively translucent, and continued to grow well after at least 2 subculture passages on similar media.

Transfer of *in vitro* tissue, predominantly callus, that originated on media containing various plant growth regulators, including BA, 2,4-D, NAA, TDZ, and picloram, to 1/2 MS only, did not induce shoot organogenesis after 21-28 days. Shoot growth occurred, presumably from pre-existing meristems, some prolific growth of brown, non-meristematic callus took place, and some white, friable callus developed on tissue from NAA x BA, after 21 days. In most cases, viability gradually decreased over time.

Attempts to transfer callus from M1 1, M1 0.5, and M1, to 1/2 MS plus 90 g L^{-1} sucrose, 10% (v/v) coconut water, and 10 μM 2,4-D were not very successful due to fungal contamination. No cultures lasted beyond 14 days at which time callus growth was negligible.

2.2.4.2 Subcultures

In subculture round I, only materials from selected levels of plant growth regulator factorial trial I were subcultured, particularly those that produced vigorous growth initially. Shoot organogenesis was not achieved after 28 days. Shoot initiation occurred at several levels, presumably from pre-existing meristems. Vigorous callus growth occurred at most levels, producing white, green, dark green, red, pink, pinky-brown and dark red callus. Bright green callus was the most vigorous. Callus morphology included friable, nodular and smooth types. Most of these cultures were not subcultured further.

In subculture round II the majority of cultures came from plant growth regulator factorial trial I and a few from subculture round I. For the majority, the response after 28 days was very similar to round I, shoot organogenesis was not achieved and callus growth was vigorous at most levels although colour variation was not as evident, with green predominating for most cultures. Cultures from round I, which had undergone 2 subcultures, lost viability after 21 days and did not survive.

In subculture round III, all cultures came from round II and had now undergone 2 subcultures. These began to lose vigour from the outset and after 7 days most were losing viability. Higher levels of contamination were evident in this trial than in previous subcultures. By day 28 all cultures were dead.

Root tips from 1/2 MS plus 2.26 μM 2,4-D and no BA, that were subcultured onto identical media survived for approx. 5 months. From original explants which were brown in colour, root hairs formed, nodular swellings appeared and numerous green cells proliferated. After 17 weeks the green cells had largely disappeared and the cultures became static. Shoot organogenesis was not achieved.

2.2.4.3 Induction

Callus produced from cotyledons during the direct shoot regeneration trial included vigorous bright green, slow green, and nodular types. Contamination rates were high and the resultant over-sterilisation of some explants led to their becoming non-viable. Callus initiation occurred by day 7 but shoot organogenesis was not achieved after 6-7 weeks.

On medium D4 0.5, (8.88 μM BA x 2.26 μM 2,4-D) cotyledons produced callus after 14 days, and on medium D5 2.0 (22.2 μM BA x 9.04 μM 2,4-D) callus was evident after 7 days. Callus types included slow dark, vigorous bright green, friable, and white filamentous. Shoot organogenesis was achieved in neither case after 28-35 days.

When whole immature seeds were exposed to media containing factorial concentrations of BA & 2,4-D, callus initiation occurred after 7 days at nearly every level of treatment. After 28 days, callus growth was evident at most levels although lower BA concentrations generally induced callus more effectively (Figure 2). In general, a different type of callus was produced with these explants. White, filamentous, friable callus tissue, often originating at the micropyle, was usually predominant as opposed to the smooth, green, dense callus that had been produced previously from other types of explant. At some levels, roots and hairs formed but after 8-9 weeks shoot organogenesis was not achieved.

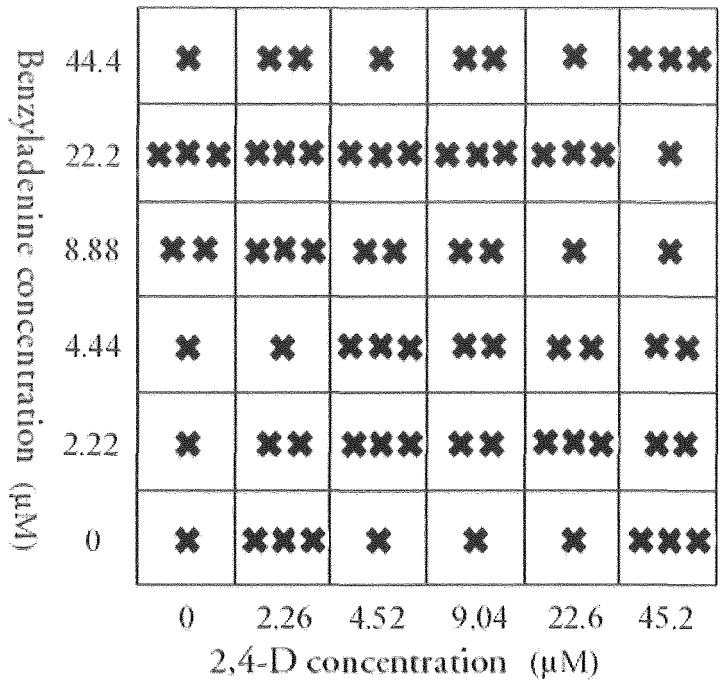


Figure 1. Callus induction response of *Chamaecytisus palmensis* cotyledons, derived from germinated mature seeds, on 1/2 MS plus BA x 2,4-D after 28-35 days.
✖ = minimal ✖✖ = moderate ✖✖✖ = vigorous (n=2)

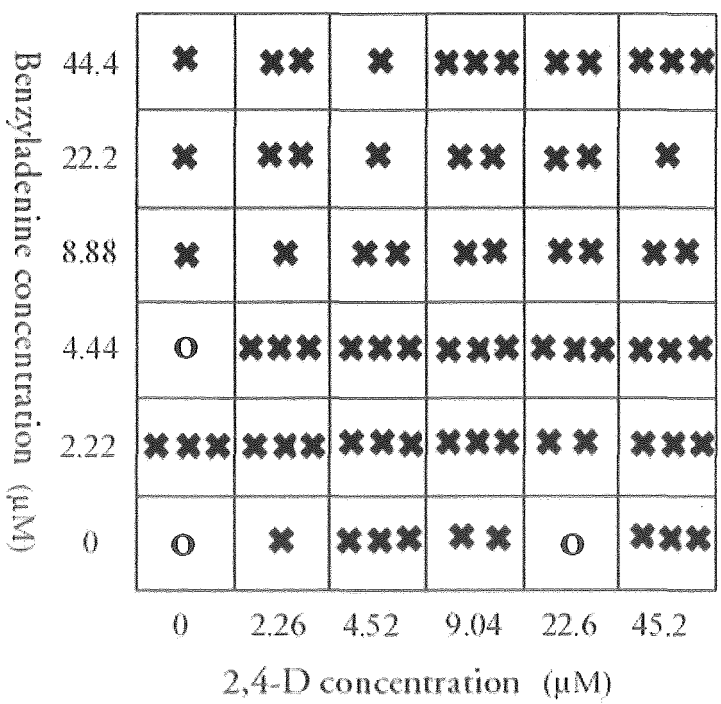


Figure 2. Callus induction response of *Chamaecytisus palmensis* whole immature seeds on 1/2 MS plus BA x 2,4-D after 28 days.
O indicates nil callus formation.
✖ = minimal ✖✖ = moderate ✖✖✖ = vigorous (n=3)

Some of the embryos germinated and produced shoots which were removed from the cultures as they appeared.

When cotyledonary nodes were cultured on media containing semi-factorial combinations of NAA x BA, minimal callus was produced after 7 days. After 38 days substantial callus growth was evident at 11.1 μM BA x 13.4 μM NAA, 2.22 μM BA x 26.9 μM NAA, and 22.2 μM BA x 26.9 μM NAA. Predominantly green nodular callus was produced. After 12 weeks the cultures were all still healthy and material from them was subcultured to the same medium, where it remained viable for a further 7 weeks after which it was transferred to either 1/2 MS, or 1/2 MS plus TDZ & picloram. Shoot growth, presumably from pre-existing meristems, and limited root initiation occurred but *de novo* shoot organogenesis was not achieved.

When shoot tips from *in vitro* plants were excised and cultured separately on 1/2 MS plus TDZ & picloram, minor contamination was evident in nearly all the cultures from the outset, despite the fact that the source cultures appeared to be pathogen free. The recently developed contamination-control product, Plant Preservative Mixture (PPM), was used with limited success in attempts to control the contamination. Although the cultures never became completely pathogen free, most of the cultures remained viable throughout. After 7 days limited amounts of very slow growing callus had formed with numerous hairs protruding, along with some expansion of the explant. At 28-35 days, several cultures started producing green nodular callus, similar to what had been seen previously with other types of explant, although much slower growing and still with hairs protruding. At 50 days one more culture started producing the same sort of green, nodular callus on differentiated tissue. Shoot organogenesis was not achieved.

On medium containing 90 g L^{-1} sucrose, plus auxin (2,4-D or picloram), explants, including cotyledons, hypocotyls, and roots, were slow to produce callus. Cotyledons performed the best, producing modest amounts of callus after 14 days in most cases, and strong callus growth after 21 days in a few cases. 1/2 MS plus 90 g L^{-1} sucrose alone, produced predominantly expansion of the explant and very little callus. Callus types produced after 7 days on 4.14 μM picloram included white, pink, and filamentous. After 25 days on 4.14 μM picloram, 4.52

μM 2,4-D, or 9.04 μM 2,4-D, callus colours ranged from green, white, pink, red, clear to translucent, and morphologies from spherical, filamentous, friable to nodular (Plate 2C-F & Plate 3). After 28 days, growth rates appeared to slow down. Shoot organogenesis was not achieved. When callus from these cultures was transferred to media C2.0, C3.3, (C), (D), or M1, viability decreased very rapidly in most cases and the cultures did not survive.

2.2.5 Clonal shoot multiplication *in vitro*

Initially shoots and callus that arose on plant growth regulator factorial trial I were subcultured onto similar media for up to 10 weeks. In the early stages (7-28 days) shoot growth was minimal, some shoots were quite stunted and many grew very slowly. From week 5 they began to improve, the number of shoots began to increase and many became quite vigorous. At week 10 the best performing shoots were subcultured to similar media where they grew vigorously from the outset. After 28 days, some of the culture media on which the shoots were growing, began to change colour from clear/translucent to a pinky-brown colour presumably due to the release of phenolics into the medium. From this point, all fresh shoot multiplication media was 1/2 MS only. Additional *in vitro* shoot material came from; (1) transfer to 1/2 MS, (2) NAA x BA trial, (3) immature seeds on BA x 2,4-D.

Originally 8 separate clonal lines were established, seven of which had foliage with a 'wet' appearance *in vitro* from the outset, due to hyperhydration. The wet clones were characterised by a bright green, moist-looking epidermis, with loosely-arranged surface hairs (Plate 4A). The other clone, #7, had from the outset, dry, dull, dark green foliage *in vitro*, with closely appressed surface hairs, which closely resembled *in vivo* foliage. After approx. 1-2 months, 2 of the 'wet' clones lost viability and died.

Hyperhydrated clones always caused the culture media to change gradually from a clear, translucent colour, to a brown, semi-opaque colour over several weeks. Clone #7 never caused the media to change colour. Even after several months, the agar in jars containing clone #7 was still the same colour as when it was first poured (Plate 4B).

PLATE 2 Mature *Chamaecytisus palmensis* seeds, and *in vitro* callus cultures of various ages, derived from *Chamaecytisus palmensis* cotyledons, on 1/2 MS medium containing a variety of plant growth regulators.

(A) Mature *Chamaecytisus palmensis* seeds illustrating the arrangement of the aril in relation to the testa.

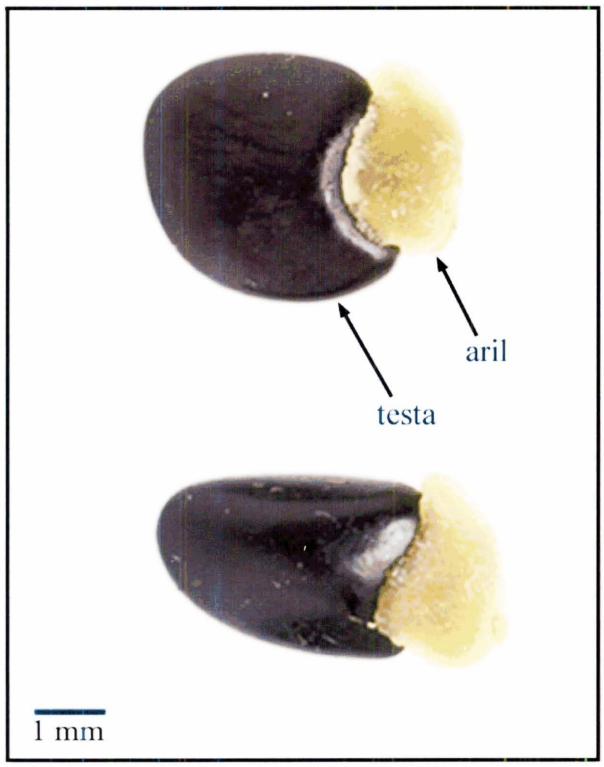
(B) Light green, compact callus growth on 1/2 MS plus 4.54 μM TDZ (top) and darker, less dense callus growth on 1/2 MS plus 4.54 μM TDZ & 4.14 μM picloram (bottom).

(C) Arrows indicate (1) newly formed green, nodular callus cells amongst white and pink translucent callus cells, and (2) the original cotyledon tissue.

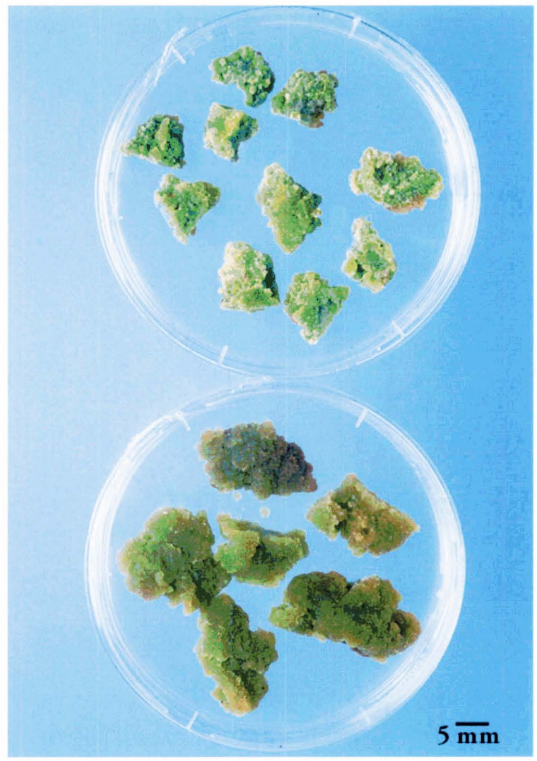
(D) Arrows indicate (1) light green spherical callus cells proliferating in interior of cell mass, and (2) white filamentous callus cells occurring in outer layers.

(E) Arrows indicate (1) tight, spherical mass of dense pink nodular callus cells, (2) white, translucent callus cells, and (3) green, nodular callus cells.

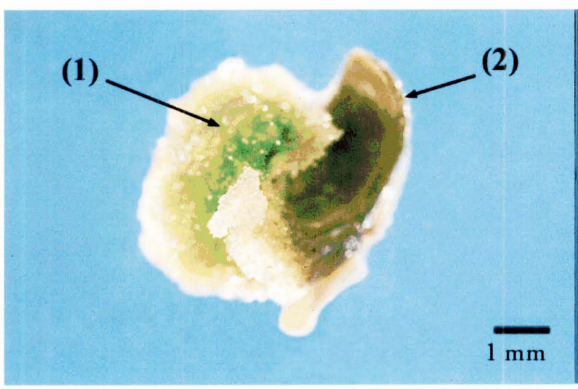
(F) Arrows indicate (1) pink and white translucent callus cells proliferating at periphery, and (2) white, thread-like filamentous callus cells occurring at apex of cotyledon.



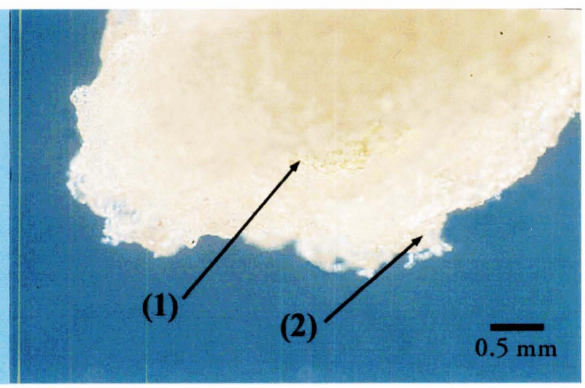
(A) Mature *Chamaecytisus palmensis* seeds.



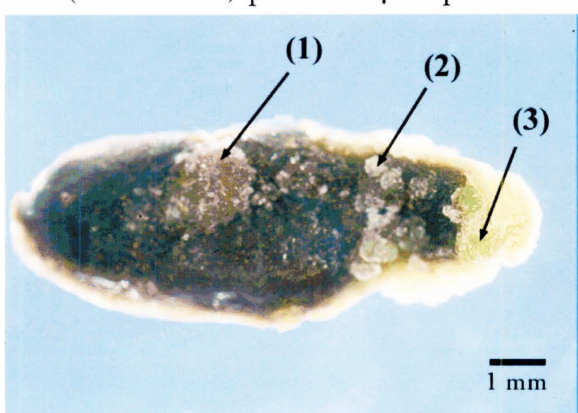
(B) 3 month-old callus cultures of *Chamaecytisus palmensis*.



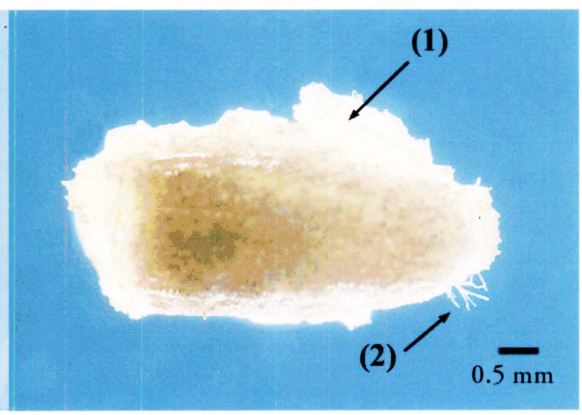
(C) Callus development on *Chamaecytisus palmensis* cotyledon after 25 days on 1/2 MS (9% sucrose) plus 4.14 µM picloram.



(D) Callus development on *Chamaecytisus palmensis* cotyledon after 25 days on 1/2 MS (9% sucrose) plus 4.14 µM picloram.



(E) Callus development on *Chamaecytisus palmensis* cotyledon after 25 days on 1/2 MS (9% sucrose) plus 4.52 µM 2,4-D.



(F) Callus development on *Chamaecytisus palmensis* cotyledon after 7 days on 1/2 MS (9% sucrose) plus 4.14 µM picloram.

PLATE 2

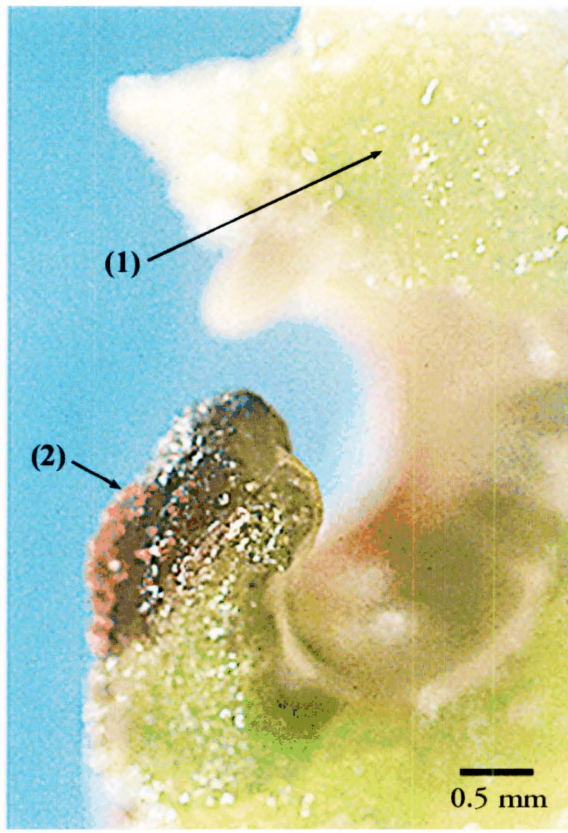
PLATE 3 *In vitro* callus cultures of various ages, derived from *Chamaecytisus palmensis* cotyledons, on 1/2 MS medium with and without a variety of plant growth regulators.

(A) Arrows indicate (1) bright green, nodular callus cells, and (2) red pigmented spherical callus cells.

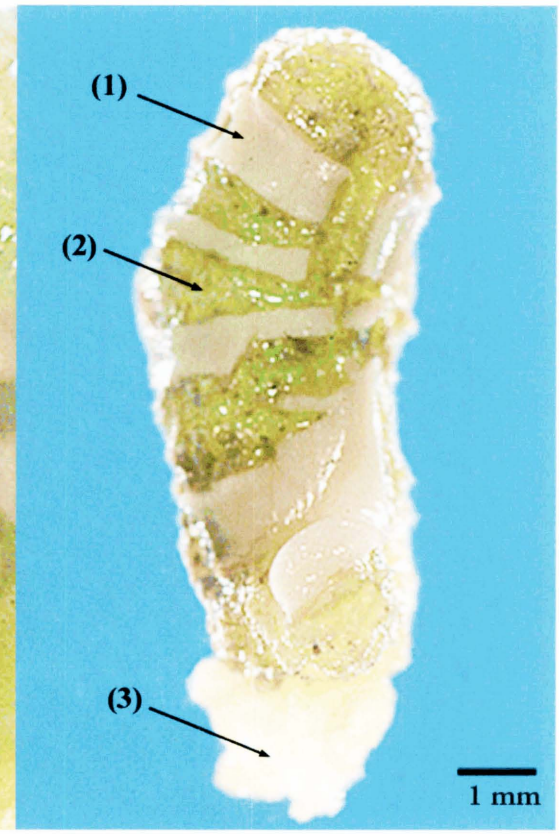
(B) Arrows indicate (1) bands of epidermal cells derived from original cotyledonary tissue, (2) proliferating green meristematic callus cells, and (3) mass of cream coloured compact callus cells at apex.

(C) Arrows indicate (1) light green compact nodular callus cells, and (2) pink and white filamentous callus cells.

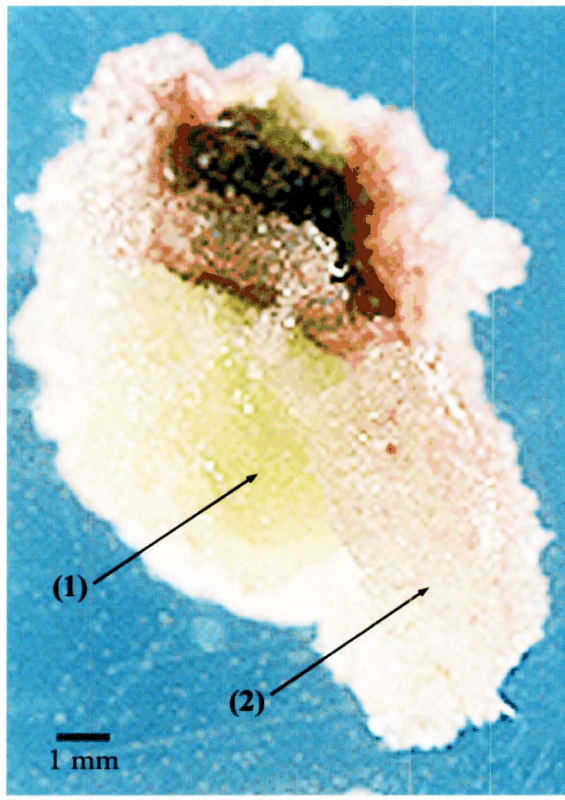
(D) Arrow indicates (1) white, thread-like filamentous callus cells occurring at apex of cotyledon.



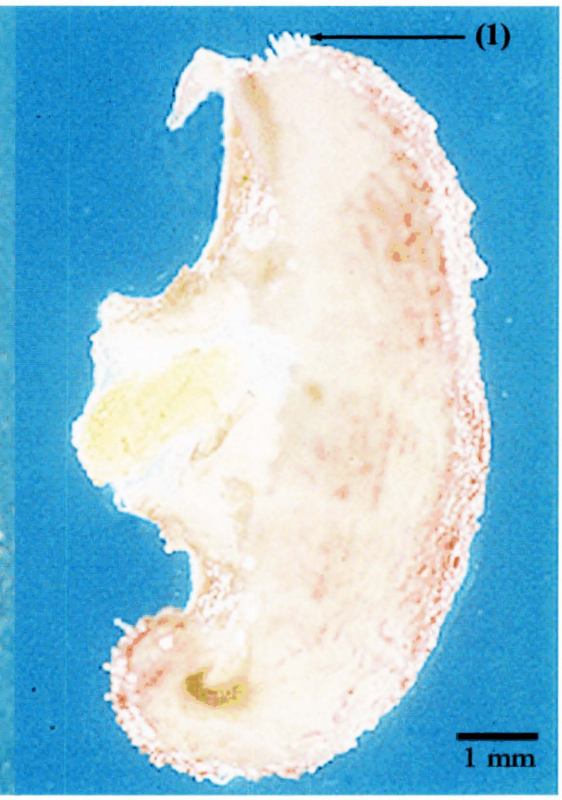
(A) Callus formation on *Chamaecytisus palmensis* cotyledon after 25 days on 1/2 MS (9 % sucrose) plus 9.04 mM 2,4-D.



(B) Callus formation on *Chamaecytisus palmensis* cotyledon after 28 days on 1/2 MS medium (9 % sucrose).



(C) Callus development on *Chamaecytisus palmensis* cotyledon after 25 days on 1/2 MS (9% sucrose) plus 9.04 mM 2,4-D.



(D) Callus development on *Chamaecytisus palmensis* cotyledon after 7 days on 1/2 MS (9% sucrose) plus 4.14 mM picloram.

PLATE 3

After 11 months in continuous culture, normal foliage was spontaneously produced by clone #4. This only happened spontaneously in a small proportion of clone #4 cultures, less than 5 % (Plate 4C-E). When explants derived from this normal foliage were subcultured to fresh 1/2 MS, they retained their dry morphology. Normal shoots, when subcultured, always performed better than hyperhydrated shoots, growing more vigorously and encountering fewer problems with contamination. Both normal and hyperhydrated shoots were subcultured *in vitro* for more than 2 years with no tendency to lose viability over that time.

2.2.6 Propagation of *C. palmensis* clones

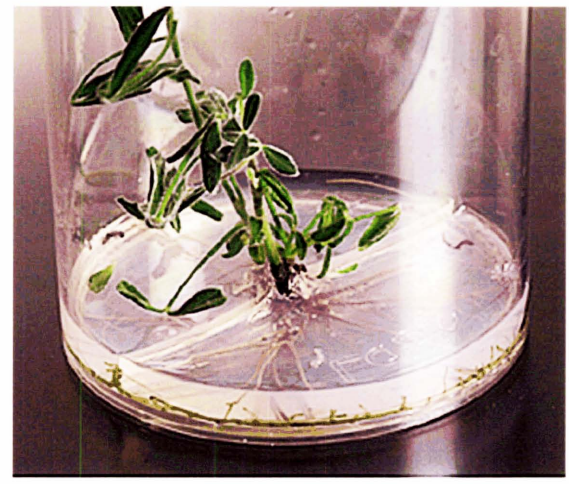
Roots initiated spontaneously *in vitro* in all of the clonal lines. In the hyperhydrated clones roots arose sporadically, whereas in clone #7, every individual shoot formed roots within approx. 10 days of being placed on the medium. The roots that formed on #7 were thicker, more vigorous and more numerous than those that formed on the hyperhydrated shoots (Plate 5). Roots that formed on hyperhydrated shoots were usually quite thin and brown in colour, with bulbous apices, whereas normal-shoot roots were initially much paler, almost white, and had pointed ends. When clone #4 started producing normal shoots, it was hoped, as in the case of clone #7, that every subcultured shoot would form roots rapidly. This did not eventuate and root formation on normal, subcultured #4 shoots remained sporadic.

When the rooted shoots were moved to *ex vitro* conditions, the normal shoots acclimatised more rapidly than the hyperhydrated shoots, requiring less humidity control, for a shorter time. Typically, 1-2 weeks acclimatisation was required for normal shoots and 2-4 weeks acclimatisation for hyperhydrated shoots. Hyperhydrated shoots were also more prone to fungal contamination when moved to *ex vitro* conditions, usually requiring the application of a fungicide. When rooted hyperhydrated shoots were established under standard glasshouse conditions, after a few weeks these clones produced normal-looking leaves.

Attempts to induce roots on wet clone shoots *in vitro* with 1/2 MS plus 4.92 μM IBA were unsuccessful after 7 weeks, when not even sporadic roots had appeared.

PLATE 4 *Chamaecytisus palmensis* clonal *in vitro* shoot cultures illustrating aspects of hyperhydrated and normal foliage, and lack of discolouration of agar medium by clone #4 after long term *in vitro* culture.

(A) *In vitro* culture of *Chamaecytisus palmensis* clone #1 displaying 'wet', glossy, bright green foliage due to hyperhydration.



(B) *Chamaecytisus palmensis* clone #7 *in vitro*, displaying clarity of agar medium after approx. 8 months in culture.

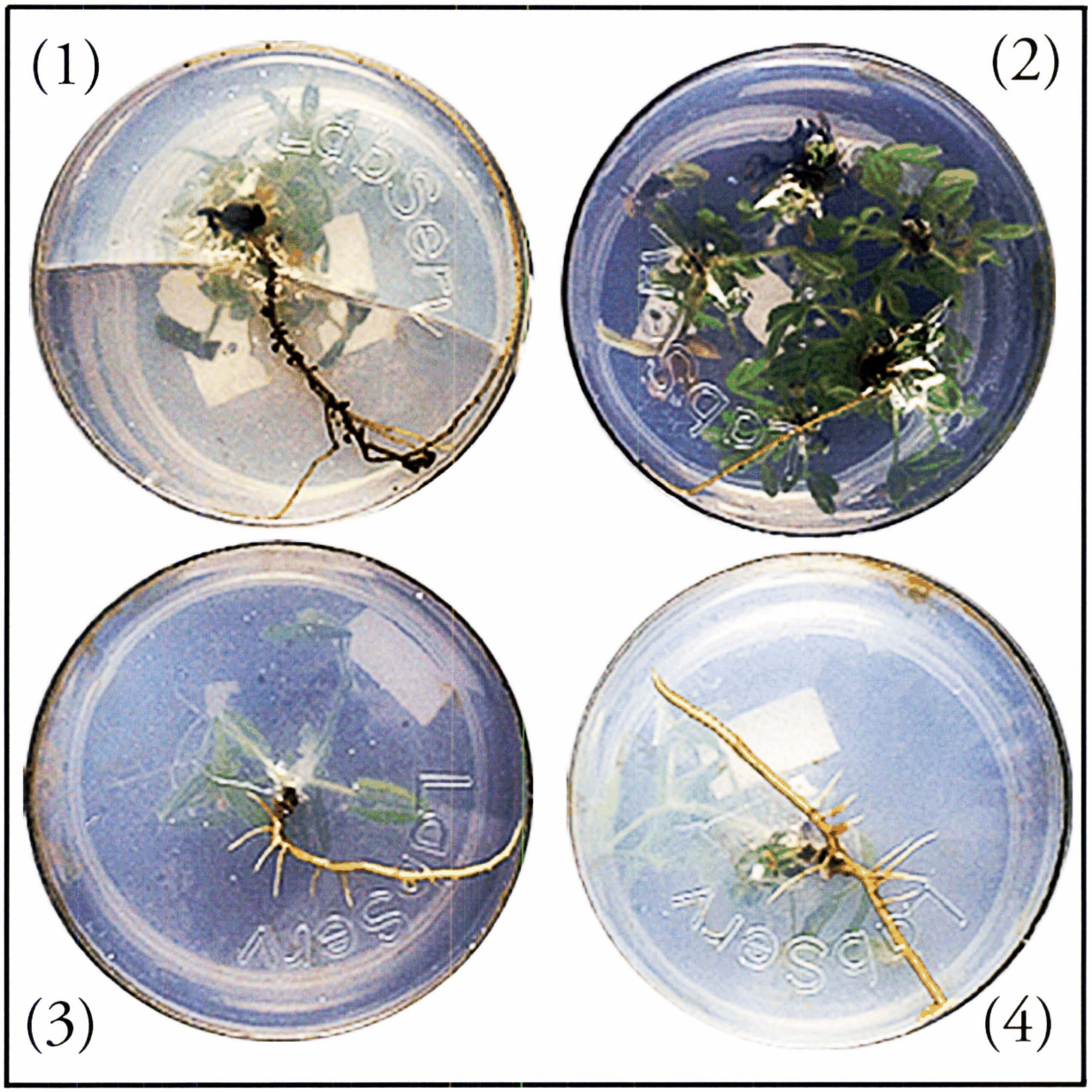
(C) *Chamaecytisus palmensis* clone #4 *in vitro* exhibiting hyperhydrated foliage (left), and normal foliage (right).

(D) Hyperhydrated foliage typical of *Chamaecytisus palmensis* clone #4 *in vitro.*

(E) Normal foliage of *Chamaecytisus palmensis* clone #4 produced after 11 months in culture.

PLATE 4

PLATE 5 *Chamaecytisus palmensis* clonal *in vitro* root formation illustrating morphological differences in newly-formed roots, between clone #7 and other clonal lines. Roots forming on clone #7 are thicker, lighter coloured, and more vigorous than those forming on other clonal lines.



In vitro root formation in *Chamaecytisus palmensis* on 1/2 MS media. (1) Clone #4 after 3 months. (2) Clone #2 after 8 weeks. (3) & (4) Clone #7 after 4 weeks.

PLATE 5

2.3 Change of *C. palmensis* callus fresh weight

The medium chosen for determining callus fresh weight gain was selected on the basis of the results from factorial plant growth regulator trial I. Callus induction and growth on 1/2 MS plus 22.2 μM BA were as vigorous as on any of the other combinations and therefore presumed to be representative of callus growth potential within this system. Figure 3 depicts the trend over 28 days and shows a near linear relationship between growth rate and time.

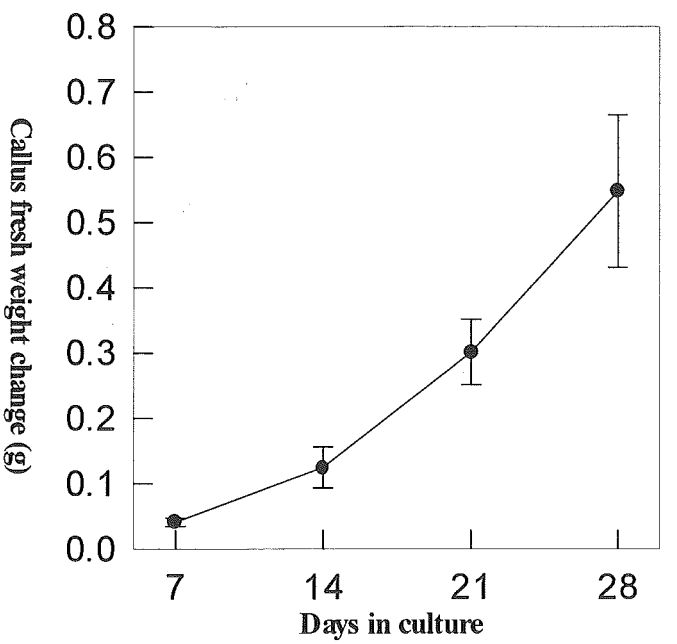


Figure 3. Change in fresh weight of *Chamaecytisus palmensis* callus formedfrom cotyledons on 1/2 MS plus 22.2 μM BA. Bars represent standard error (n=3).

(3) LEAD EFFECTS

3.1 *P. radiata* 14 day-old seedlings in lead nitrate solution: morphological effects

In the 1208 μM Pb trial, three days after treatment commenced the majority of the seedlings had fully expanded cotyledons and the true shoot had extended to approximately 1 cm in length. At this stage controls and treatments showed similar growth rates. By 7 days, the Pb-treated seedlings were beginning to look stressed and were lagging behind the controls which were vigorous and healthy. Control shoot tips had extended to approximately 2 cm in length but treated seedling shoot tips were still only approximately 1 cm. After 9 days, the treated seedlings looked stunted compared to the controls and there was practically no lateral root formation whereas lateral root formation on controls was pronounced, with roots up to 3 cm in length being produced (Table 1).

Table 1. Number of lateral roots initiated on *Pinus radiata* seedlings after 9 days in 1208 μM $Pb(NO_3)_2$ and dH_2O control.

Replicate	1	2	3	4	5	6
Control	5	6	4	7	0	3
Pb	0	0	1	0	0	0

The roots of the treated seedlings were a uniform dark brown colour whereas the control roots displayed a variety of colours including white, pink, and light brown. At the conclusion, after 16 days, when treated plant samples were examined with EPXMA, Pb deposition was not detected.

When seedlings were exposed to multiple Pb concentrations for approx. 6 weeks, the health and vigour of the plants deteriorated in relation to the Pb concentration and the length of time exposed. At 151 μM Pb, plants showed no signs of stress for 3 weeks and then only minimally. At 302 μM Pb, plants showed stress after 10 days and at 604 μM Pb plants showed stress at 6 days. Plants exposed to 1208 μM Pb were displaying signs of stress after 3 days. Controls in

dH_2O remained healthy throughout and continued to grow whereas seedlings exposed to 1208 μM Pb had all collapsed at the conclusion (Table 2).

Table 2. Average response of *Pinus radiata* seedlings to $Pb(NO_3)_2$ in solution over time (n=5).

(+ + + = Healthy + + 0 = Stressed + 0 0 = Losing viability 0 0 0 = collapsed)

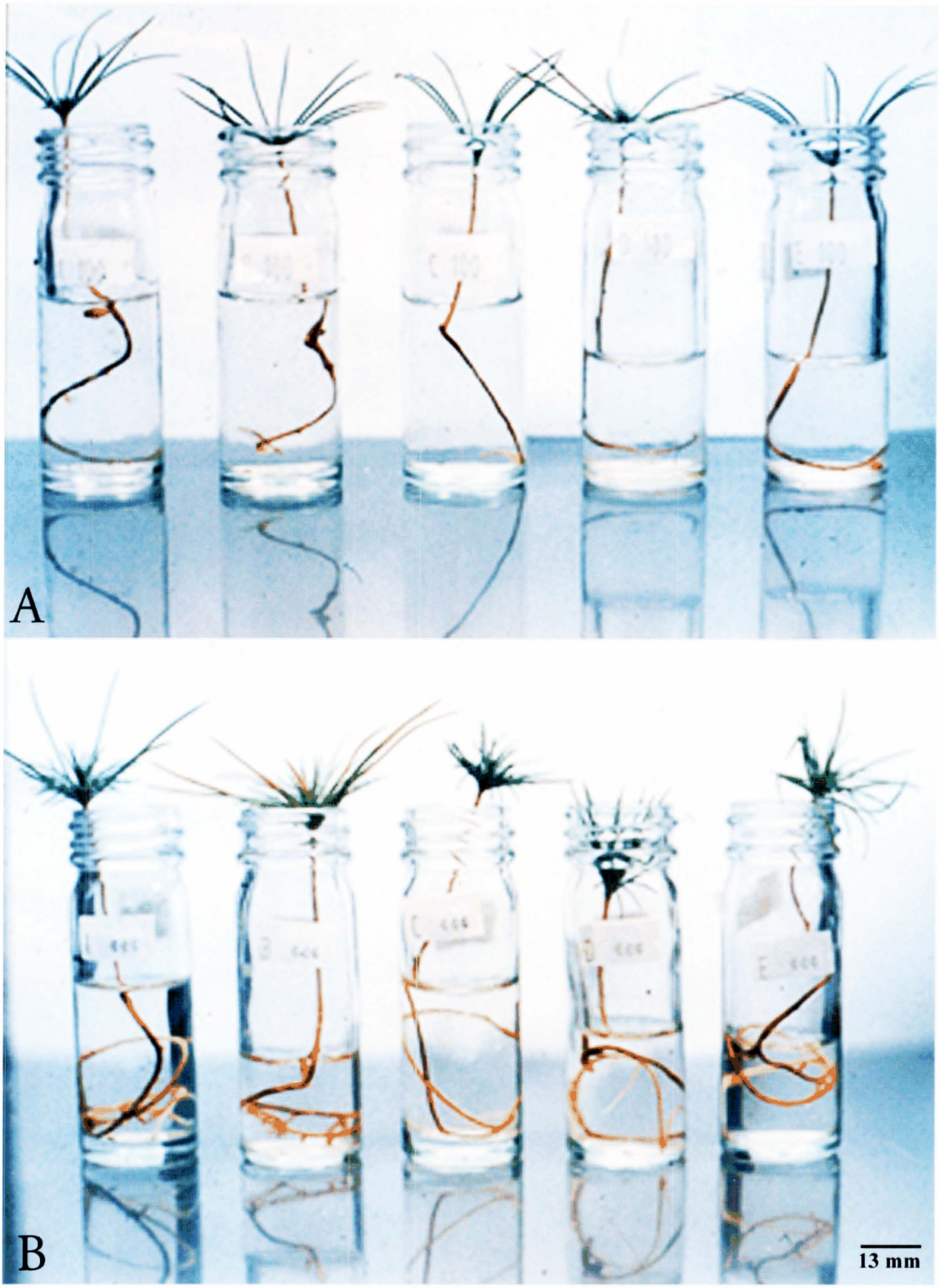
WEEK	1	2	3	4	5	6
dH_2O	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
151 μM	+ + +	+ + +	+ + 0	+ + 0	+ + 0	+ + 0
302 μM	+ + +	+ + 0	+ + 0	+ 0 0	+ 0 0	+ 0 0
604 μM	+ + 0	+ 0 0	+ 0 0	+ 0 0	+ 0 0	+ 0 0
1208 μM	+ 0 0	+ 0 0	+ 0 0	+ 0 0	+ 0 0	0 0 0

Roots exposed to Pb turned dark brown compared to the controls which remained white or pale in colour. Treated roots also began to become generally constricted and narrower, relative to the controls, 5 days after being exposed to Pb. Many roots exposed to Pb also developed localised constrictions for 2-3 mm that severely narrowed the diameter of the root, up to 50 %. This effect was more prevalent at higher Pb concentrations. Shoot growth was also retarded by exposure to Pb as shown in Plate 6. The control seedlings had well developed root systems with numerous lateral roots developing and shoot growth approaching 2 cm in length whereas in 302 μM Pb, seedlings had poorly developed root systems with negligible lateral root development and minimal shoot growth.

3.2 *P. radiata* and *C. palmensis* seedlings in lead nitrate solution: Fresh weight changes

After 14 days, the gain in fresh weight of *P. radiata* seedlings was greatest at the 20 μM Pb treatment level (Figure 4a).

PLATE 6 Root formation in *Pinus radiata* seedlings after 48 days in solution culture, illustrating how Pb treatment has suppressed root elongation, lateral root formation, and shoot growth.



Pinus radiata seedlings after 48 days in solution culture.
(A) 302 µM lead nitrate. (B) Distilled water.

PLATE 6

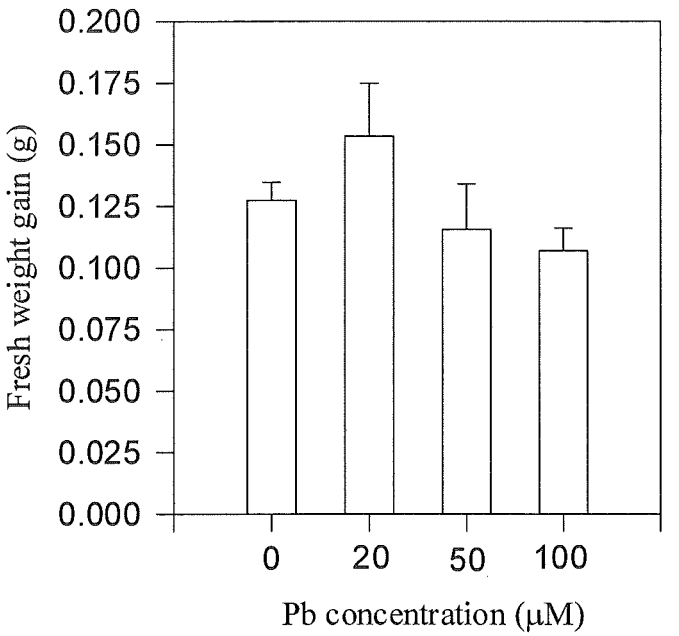


Figure 4a. Fresh weight gain of 26 day-old *Pinus radiata* seedlings in lead nitrate solution at pH 4.5 after 14 days. Bars represent standard error (n=3).

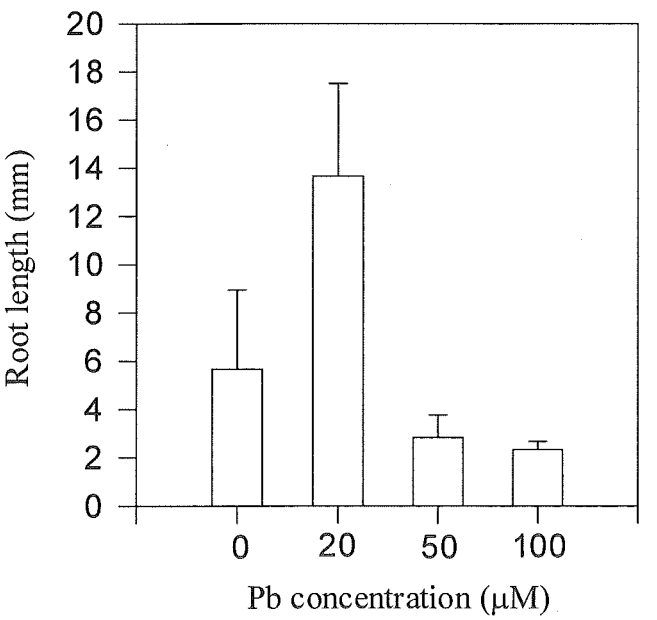


Figure 4b. Length of longest lateral root developing on 26 day-old *Pinus radiata* seedlings after 14 days in lead nitrate solution. Bars represent standard error (n=3).

The data presented here is for one round and is representative of the 3 rounds conducted. The 50 μM Pb and 100 μM Pb treatment levels produced less fresh weight gain than the control. Similarly the 20 μM Pb treatment produced the longest *P. radiata* lateral root after 14 days (Figure 4b). The control produced the second longest lateral root and the 50 μM Pb and 100 μM Pb treatments produced lateral roots of approximately the same length.

After 14 days treatment, uniform 26 day-old *C. palmensis* seedlings produced the greatest fresh weight gain in 20 μM Pb, marginally ahead of the control and well ahead of those in 50 and 100 μM Pb (Figure 5a). The data presented here is for one round and is representative of the 3 rounds conducted. For lateral root development (Figure 5b) 0 Pb produced the longest root, followed by 20, 50, and 100 μM Pb.

3.3 *In vitro* shoots of *P. radiata* cultured in agar based medium supplemented with lead nitrate

After 42 days, all shoots from clones #2, #4, #5, and #9, at all levels of treatment were still bright green, turgid, and vigorous except for one or two replicates that were contaminated with fungal infection. In some cases, older needles gradually turned brown after a few weeks but this happened sporadically and in different Pb concentrations and there seemed to be no particular pattern in how this phenomenon manifested itself. For this reason close attention was paid to needle tips as an indicator of a response to Pb in the medium as newly formed needles should have reflected stress on the shoot. At the conclusion of the experiment practically no needle tip discolouration was observed (Plate 7). The gradual browning observed in older needles did not have any great significance attached to it as it was a characteristic response commonly seen in *P. radiata* shoots in *in vitro* culture over time. Figure 6 shows the results of combining the 3 replicates at each level of treatment and weighing them at the conclusion of the trial. Clones 2 and 4 displayed a trend of decreasing total fresh weight with increasing Pb concentration except at 100 μM Pb.

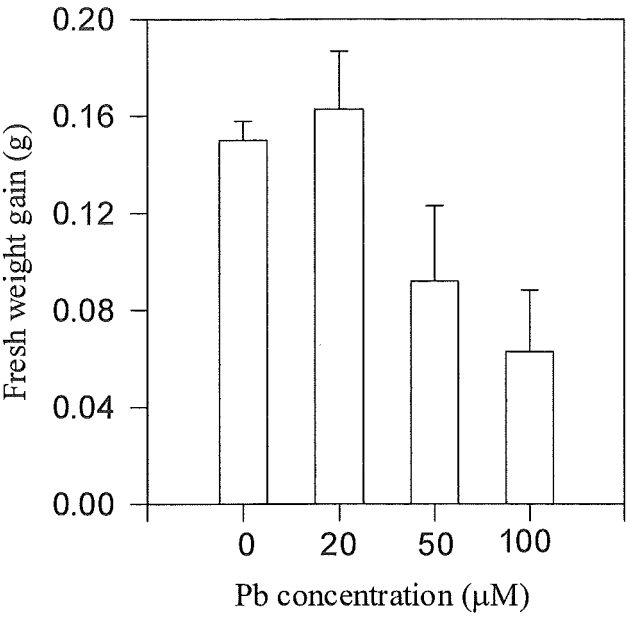


Figure 5a. Fresh weight gain of 26 day-old *Chamaecytisus palmensis* seedlings after 14 days in lead nitrate solution at pH 4.5. Bars represent standard error (n=3).

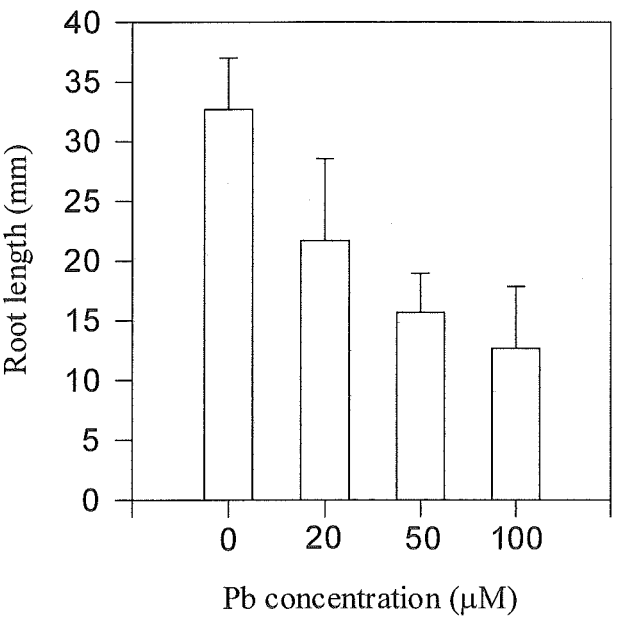
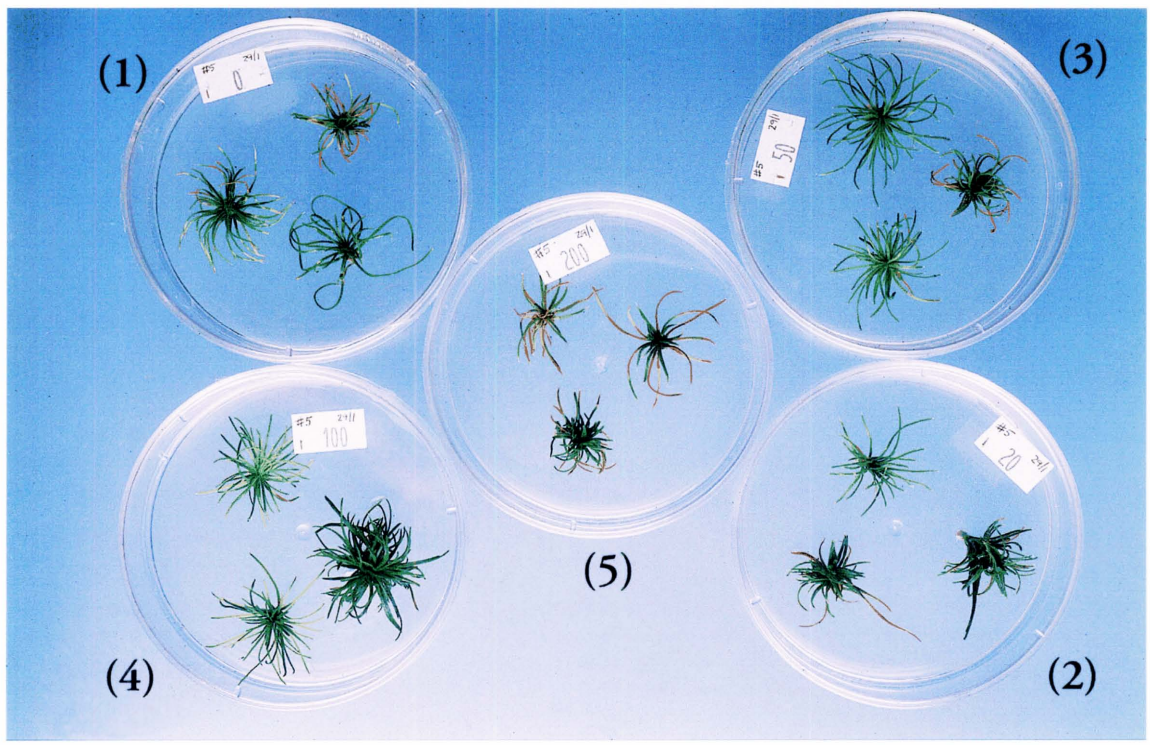


Figure 5b. Length of longest lateral root developing on 26 day-old *Chamaecytisus palmensis* seedlings after 14 days in lead nitrate solution.Bars represent standard error (n=3).

PLATE 7 Effect of Pb treatment *in vitro* on excised shoots of *Pinus radiata* clone #5, illustrating inhibition of growth due to 7 weeks Pb exposure, particularly at the 200 μM level.



Pinus radiata clone #5 *in vitro* shoots on LPO agar plus lead nitrate after 7 weeks. (1) 0 Pb. (2) 20 µM Pb. (3) 50 µM Pb. (4) 100 µM Pb. (5) 200 µM Pb.

PLATE 7

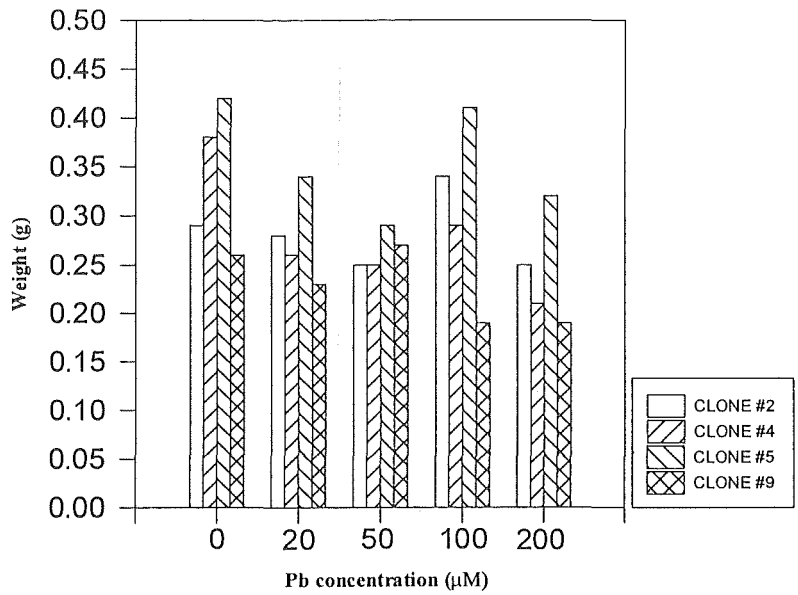


Figure 6. **Combined fresh weight of *in vitro* shoots of *Pinus radiata* clones after 7 weeks on LPO agar plus lead nitrate. One representative set of data for each clone is depicted.**

The response of clone 5 was similar except at 200 µM Pb where the fresh weight was higher than the 50 µM Pb level. Clone 9 showed decreasing fresh weight with increasing Pb concentration with a slight rise above the trend at 50 µM Pb. Two-factor analysis of variance without replication was carried out on the data depicted in Figure 6. Factor 1 was 'treatment', and factor 2 was 'clone'. The results of this analysis are presented in table 3, where it can be seen that there was a significant effect due to clones at the 99% level ($P < 0.01$).

Table 3. Analysis of variance among means of factors 'treatment' and 'clone' derived from fresh weight changes of *Pinus radiata* clones after 7 weeks on LPO agar plus lead nitrate. One representative set of data for each clone was analysed.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Treatment	0.02208	4	0.0060	3.624	0.037	3.259
Clones	0.04172	3	0.0140	9.129	0.002	3.490
Error	0.01828	12	0.0015			
Total	0.08208	19				

Unplanned multiple comparisons of means were conducted on factors 'treatment' and 'clone' using the Tukey test for equal sample sizes. Means are ranked in order of magnitude and those that are not significantly different are joined by a horizontal line.Application of the Tukey test revealed that the response of clone 5 was significantly different to the responses of clones 9, 4, and 2.

Treatment:	200	50	20	100	0
Clones:	#9	#4	#2	#5	

3.4 Effect of lead nitrate on seed germination

3.4.1 *P. radiata*

(a) In preliminary trials for this experiment, seeds were stratified for only 7 days in the dark and in most cases germination rates of 50 % or lower were recorded, regardless of $Pb(NO_3)_2$ or $NaNO_3$ concentration (Table 4).

Table 4. Germination of *Pinus radiata* seeds stratified for 7 days in dH_2O then transferred to $Pb(NO_3)_2$ solutions, or 100 µM $NaNO_3$, for 14 days.

Germ. Solution	dH_2O	20 µM	50 µM	100 µM	200 µM	$NaNO_3$
% germinated	46	40	37.5	50	30	37.5

When the stratification period was extended to 14 days in the dark, all the seeds germinated (Table 5). The data in table 5 is representative of 2 identical trials. $Pb(NO_3)_2$ up to 1000 µM does not inhibit germination of *P. radiata* seed.

Table 5. Germination of *Pinus radiata* seeds stratified for 14 days in dH_2O then transferred to $Pb(NO_3)_2$ solutions or 100 µM $NaNO_3$, for 7 days.

Solution	dH_2O	20 µM	50 µM	100 µM	200 µM	1000µM	$NaNO_3$
% germinated	100	100	100	100	100	100	100

(b) In table 6 germination results for the 4-way stratification/germination trial are represented. Stratification in $Pb(NO_3)_2$ solution does not significantly

inhibit *P. radiata* germination. Although in round I, 1000 μM Pb stratification (C and D) appeared to reduce germination slightly, in round II it had no effect.

Table 6. Germination of *Pinus radiata* seed stratified for 14 days in the dark, in either dH_2O or 1000 μM $Pb(NO_3)_2$ before being moved to continuous light for 4 weeks in either dH_2O or 1000 μM $Pb(NO_3)_2$.

Round I	**(A)**	**(B)**	**(C)**	**(D)**
Strat. solution	dH_2O	dH_2O	1000 μM Pb	1000 μM Pb
Germ. solution	dH_2O	1000 μM Pb	1000 μM Pb	dH_2O
% germinated	100	90	80	90

Round II	**(A)**	**(B)**	**(C)**	**(D)**
Strat. solution	dH_2O	dH_2O	1000 μM Pb	1000 μM Pb
Germ. solution	dH_2O	1000 μM Pb	1000 μM Pb	dH_2O
% germinated	90	100	100	100

3.4.2 Multiple species

In light of the insensitivity to high Pb levels displayed by *P. radiata* during germination, it was decided that a study of the responses of other species to Pb during germination should be undertaken for the purposes of comparison.

None of the species tested required stratification but *C. palmensis* required boiling to overcome seed coat-imposed dormancy. Preliminary trials showed that wheat, corn, and clover required surface sterilisation in addition to *C. palmensis,* otherwise fungal pathogens became established, inhibited germination and obscured results despite the presence of 1000 μM $Pb(NO_3)_2$. Table 7 displays the representative germination response of the 8 species tested and also shows whether or not root tip discolouration occurred as a result of exposure to Pb. Some species, such as *C. palmensis,* naturally produce varying amounts of pigmentation in the root cap zone, and this sometimes appeared to have been caused by exposure to Pb, as this was absent in the control. There was little or no difference in the percent germination of radish, lettuce, tree lucerne and Norway spruce seeds in the presence or absence of 1000 μM $Pb(NO_3)_2$ in the germination solution. In the presence of Pb however, corn germination was 34% higher.

Table 7. Representative germination response of several plant species after 7 days (tree lucerne 22-26 days, Norway spruce 14 days) in either dH_2O or 1000 μM $Pb(NO_3)_2$.

Seed type	No. of seeds	1000 μM Pb	No. germ.	% germ.	Root tip colour
radish	30	-	30	100	-
	20	+	20	100	+
clover	60	-	29	48	-
	60	+	11	18	-
lettuce	90	-	76	84	-
	90	+	84	93	+
corn	30	-	19	63	-
	30	+	29	97	-
ryegrass	30	-	29	97	-
	30	+	15	50	-
wheat	30	-	22	73	-
	30	+	17	57	-
tree lucerne	120	-	97	81	-
	120	+	102	85	-
Norway spruce	20	-	14	70	-
	20	+	16	80	+

The germination of clover, ryegrass, and wheat seeds was inhibited by 1000 μM $Pb(NO_3)_2$. Ryegrass was the most sensitive with a 47% decrease in germination, followed by clover with a 30% decrease and wheat with a 16% decrease.

Figure 7a depicts root and shoot elongation in radish, clover, lettuce, and corn after 7 days in dH_2O or 1000 μM $Pb(NO_3)_2$. The data presented is derived from mean root and shoot elongation values generated from the materials described in Table 7. In each case, Pb exposure has resulted in an inhibition of early seedling growth compared to the control. For shoot growth, lettuce was the most inhibited by Pb, followed by clover, radish, and corn. For root growth, lettuce was the most inhibited by Pb, followed by radish, clover, and corn.

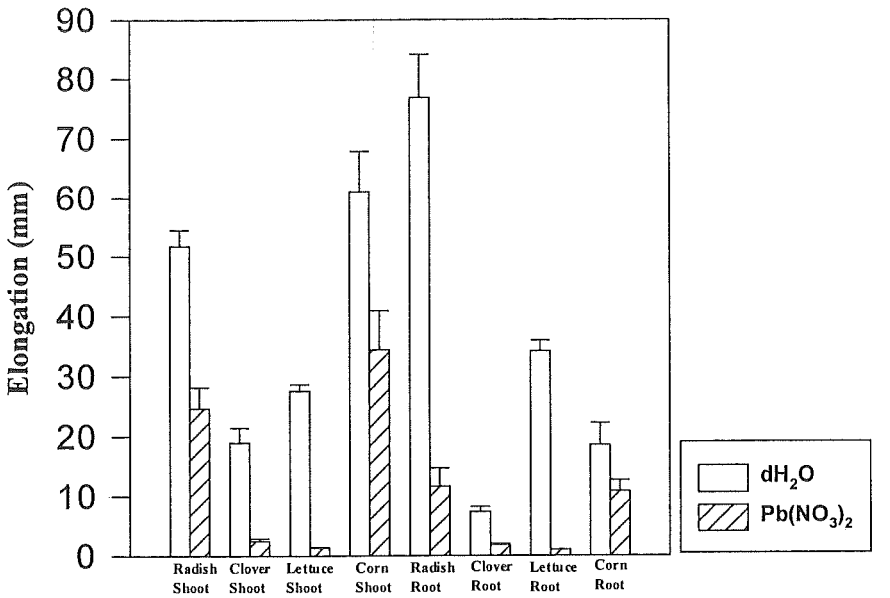


Figure 7a. Root and shoot elongation of radish (*Raphanus sativus*), white clover (*Trifolium repens*), lettuce (*Lactuca sativa*) 'Great Lakes', and corn (*Zea mays*) NK 51036 after7 days in dH2O or 1000 μM $Pb(NO_3)_2$. Bars represent standard error.

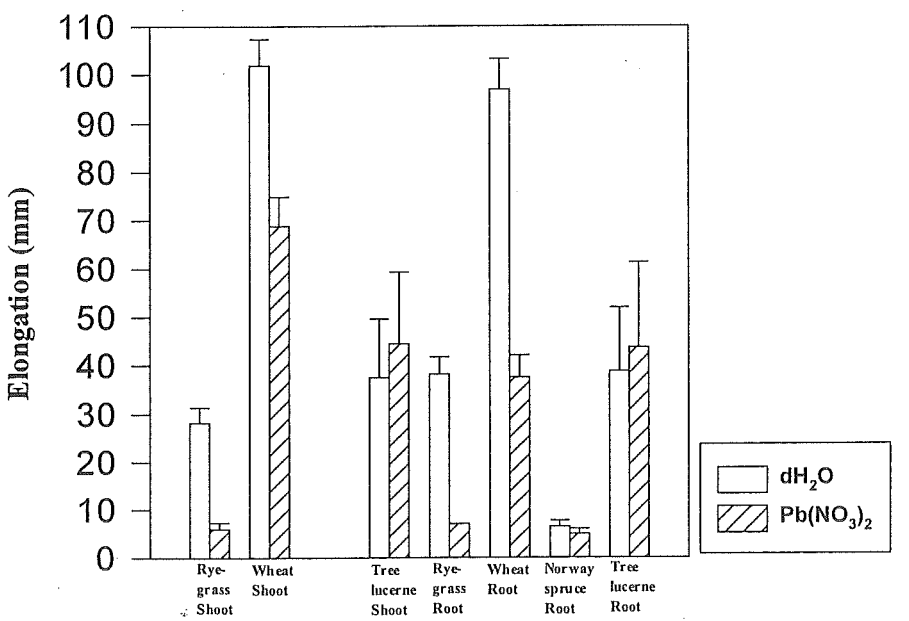


Figure 7b. Root and shoot elongation of ryegrass (*Secale cereale*) 'Ruanui', wheat (*Triticum aestivum*) 'Otane', Norway spruce (*Picea abies*) and tree lucerne (*Chamaecytisus palmensis*) after 7 days in dH_2O or 1000 μM $Pb(NO_3)_2$. Bars represent standard error.

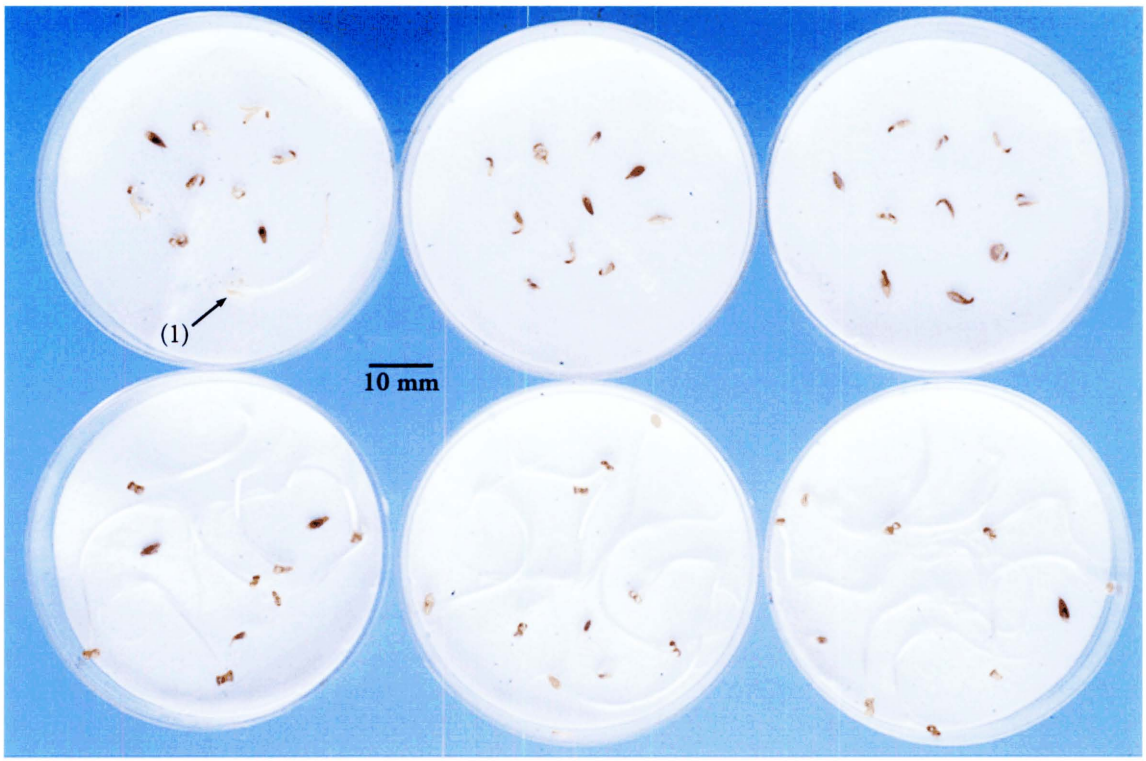
Figure 7b depicts root and shoot elongation in ryegrass and wheat, 7 days after commencement of treatment, and 14 days and 22-26 days for Norway spruce and tree lucerne respectively. The data presented is derived from mean root and shoot elongation values generated by the materials described in Table 7. Norway spruce shoot is not represented as no shoot growth had occurred after 14 days. Except for tree lucerne, Pb exposure has resulted in growth inhibition in each case, compared to the control. For shoot growth, ryegrass was inhibited most by Pb, followed by wheat. For root growth, rye grass was inhibited most by Pb, followed by wheat, then Norway spruce. In table 8, the sensitivity of seed germination, shoot elongation, and root elongation to lead exposure is summarised.

Table 8. A comparison of sensitivity to lead exposure among several plant species during seed germination and early seedling growth.
(- = insensitive, + = moderately sensitive, ++ = very sensitive, N/A = not applicable)

Species	Germination. sensitivity	Shoot elongation sensitivity	Root elongation sensitivity
Corn	-	++	+
Ryegrass	++	++	++
Wheat	+	+	++
Radish	-	+	++
Clover	++	+	++
Lettuce	-	++	++
Norway spruce	-	N/A	-
Tree lucerne	-	-	-

The species which are insensitive to Pb with regard to germination, eg., corn, radish, and lettuce, may be sensitive to Pb in other respects such as shoot and root elongation (Table 8). The extreme sensitivity of lettuce root and shoot elongation to Pb exposure is illustrated in plate 8.

PLATE 8 Effects of Pb treatment on germinating lettuce (*Lactuca sativa)* 'Great Lakes' seeds. Arrow (1) indicates seed with apparent resistance to effects of Pb during germination and early seedling growth.



Germination response after 7 days of lettuce seeds *Lactuca sativa* 'Great Lakes'.
Top row: 1000 µM lead nitrate. Bottom row: Distilled water.

PLATE 8

It is interesting to note that some lettuce seeds, approximately 1 in 30, were relatively insensitive to Pb. As illustrated in plate 8, these insensitive seeds exhibited early seedling growth in the presence of Pb, which was comparable to the controls.

When lettuce seeds that had been exposed to 1000 μM $Pb(NO_3)_2$ for 7 days were transferred to dH_2O for 7 days to assess recovery ability, seeds that displayed initial sensitivity to Pb (approximately 97 %), did not recover. No colour change or elongation occurred.

(4) LEAD UPTAKE STUDIES

4.1 Fresh weight:dry weight ratio

In determining the ratio of fresh weight to dry weight it was found that for young seedlings at least, *P. radiata* contained more water than *C. palmensis* on a fresh weight basis. Figure 8 depicts the loss in weight due to desiccation for 28 hr. at 60° C for whole seedlings, shoots, and roots. This data is summarised in Table 9, where it can be seen that for whole *C. palmensis* seedlings, approximately 20% of the original fresh weight remained after desiccation compared with approximately 14% for *P. radiata.* This difference was increased by 5% at shoot level and by 13% for roots.

4.2 Plant Pb uptake and analysis

4.2.1 Vacuum filtration in lead nitrate solution

Pb uptake levels measured by flame a.a. spectrometry in needles of *P. radiata* clones T and U after vacuum filtration in 50 and 100 μM $Pb(NO_3)_2$ for 3 hrs are shown in figure 9. It appears that Pb uptake in this manner was dependent on external Pb concentration.

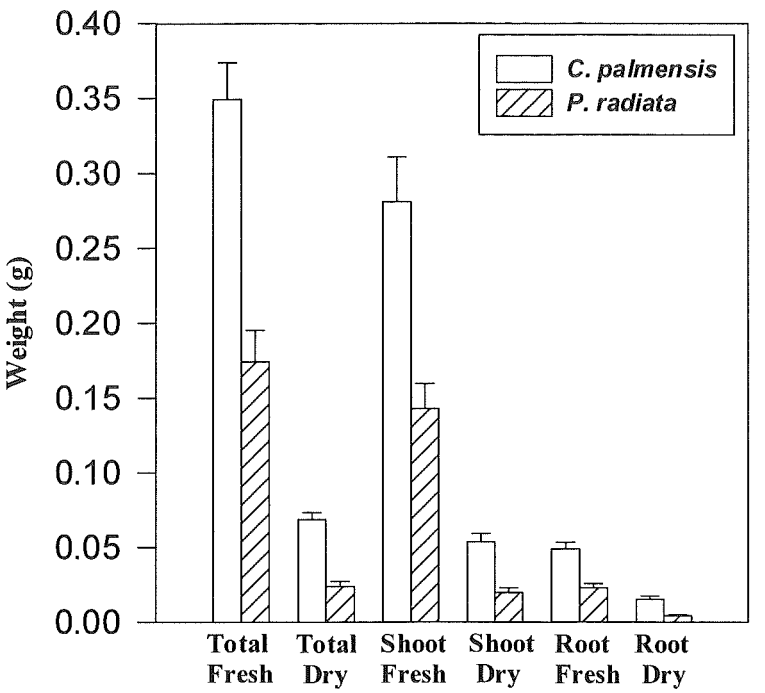


Figure 8. Fresh weight, and dry weight after 28 hr. at 60 degrees celsisus, of *Chamaecytisus palmensis* and *Pinus radiata* seedlings. Bars represent standard error (n=3).

Table 9. Dry weight of *Chamaecytisus palmensis* and *Pinus radiata* seedlings remaining after desiccation for 28 hrs. at 60° C (to constant weight), expressed as an approx. percentage of fresh weight.

	C. palmensis	*P. radiata*
Whole seedling	20 %	14 %
Shoot	19 %	14 %
Root	32 %	19 %

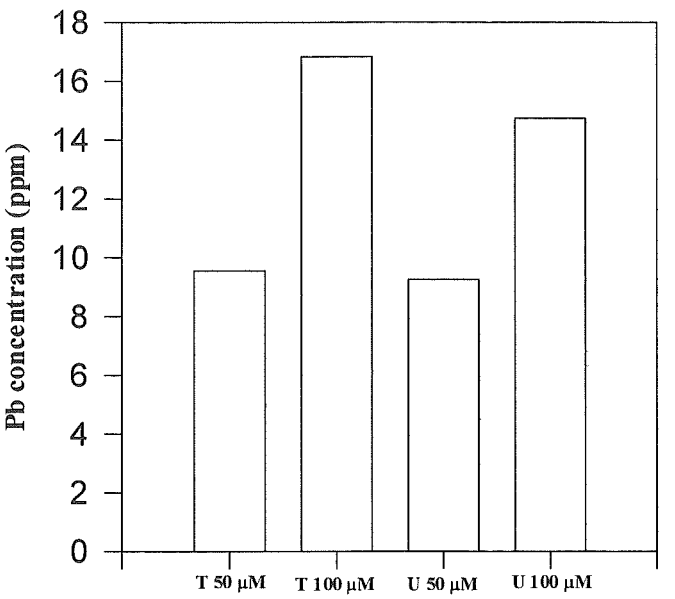


Figure 9. **Pb uptake in needles from *Pinus radiata* clones T and U, vacuum filtrated in 50 and 100 µM $Pb(NO_3)_2$ for 3 hrs. Values are derived from mean absorbances interpolated against a calibration curve.**

4.2.2 Pb uptake in soil-less media

Pb uptake from soil-less media by *Pinus radiata* seedlings at different concentrations of lead nitrate and chelators:

Initially plants were exposed to Pb for 18 days with Pb added as a single application at concentrations of up to 2000 µM, without the addition of chelating agents. Subsequently the duration of the experiments was shortened to 7 days. Pb was supplied continuously at concentrations of up to 1000 µM, and the chelating agents H-EDTA and EDTA were supplied at either 2 g kg^{-1} soil-less medium, or 0.5 g per pot. At all levels of treatment, shoot and root Pb uptake never exceeded 8 ppm which was very close to the lower limits of detection. In general root Pb uptake always exceeded shoot Pb uptake, and Pb uptake levels increased with increasing Pb treatment concentration.

Pb uptake from soil-less media by *Chamaecytisus palmensis* seedlings at different concentrations of lead nitrate:

Pb was supplied continuously for 7 days at concentrations of up to 1000 μM without the addition of chelating agents. At all levels of treatment, Pb uptake did not exceed 6 ppm which was very close to the lower limits of detection. Root Pb uptake exceeded shoot Pb uptake at all levels of treatment. Root Pb uptake levels increased with increasing Pb treatment concentration, shoot Pb uptake levels never exceeded 0.3 ppm.

4.2.3 Short-term uptake experiments

Initially, seedlings were grown in HC nutrient solution at pH 4.5 prior to treatment but later seedlings were grown in sand culture prior to treatment. When the seedlings were removed from the soil-less medium, and the roots washed in dH_2O, great care was taken not to damage root surfaces. In one trial, in addition to 20 μM $Pb(NO_3)_2$, both *C. palmensis* and *P. radiata* seedlings were exposed to 1.44 mM H-EDTA in solution for 60 min.

In all cases, after 60 min. in 20 μM $Pb(NO_3)_2$, Pb accumulation in pooled root and shoot samples of both *C. palmensis* and *P. radiata* never exceeded levels of 1.5 ppm, values which were at the lower limits of detection and regarded as unreliable. Partly as a consequence of this, 7 day exposure to Pb subsequently became the standard experimental procedure.

4.2.4 Pb uptake by seedlings grown in solution

(a) Non-aerated: In all subsequent uptake experiments, Pb treatment concentrations were either 250 μM, or 500 μM, for both *C. palmensis* and *P. radiata.* Initially, the concentration of both H-EDTA and EDTA was 1.44 mM and at this concentration one experimental round was conducted on *C. palmensis* (Figure 10). After 7 days in 500 μM Pb + 1.44 mM H-EDTA the seedlings showed symptoms of severe stress and reduced viability. Those in 500 μM Pb +1.44 mM EDTA also looked stressed, with brown mottled leaves, curled up leaves, and some leaf drop. Subsequently the chelator concentration was lowered

to 0.5 mM for both H-EDTA and EDTA. For *C. palmensis,* 5 rounds were conducted at 500 μM Pb (Figures 11 & 12; tables 10 & 11) and 3 rounds were conducted at 250 μM Pb (Figure 13; table 12). For *P. radiata,* 3 rounds were conducted at 500 μM Pb (Figure 14; table 13).

Despite lowering the chelator concentration to 0.5 mM, *C. palmensis* seedlings were still adversely affected by the chelators at this level. Compared to the controls, plants exposed to H-EDTA, and to a lesser degree EDTA, showed progressive leaf discolouration and leaf curling from the second day of treatment, beginning at the base of the plant and moving towards the apex. After this the leaves would begin to drop as they lost viability, beginning at the base and often leaving the petioles attached to the stem (Plate 9B). All shoot tips remained green and viable.

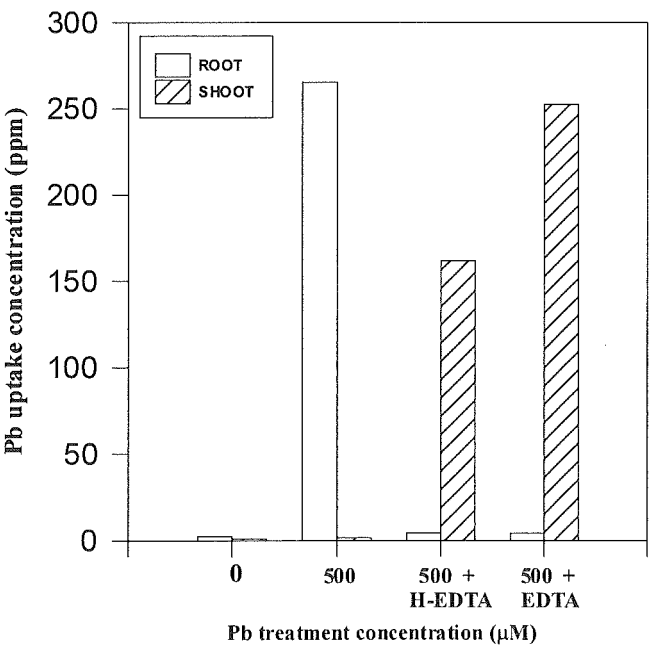


Figure 10. **Pb uptake from non-aerated solution by seedlings of *Chamaecytisus palmensis* after 7 d in $Pb(NO_3)_2$ +/- 1.44 mM H-EDTA or EDTA. Values are mean sample absorbances interpolated against a calibration curve.**

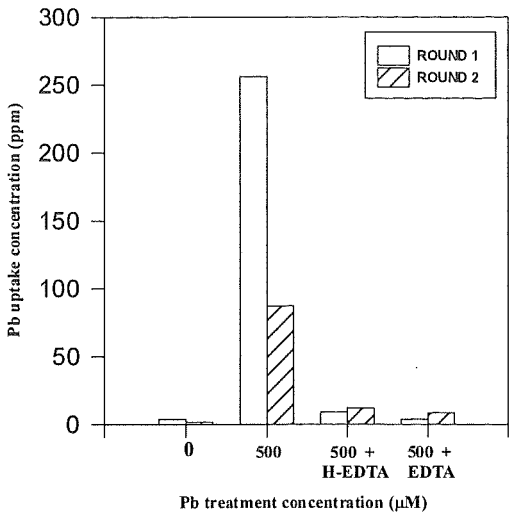


Figure 11a. Root Pb uptake from solution by seedlings of *Chamaecytisus palmensis* after 7 d in $Pb(NO_3)_2$ +/- 0.5 mM H-EDTA or EDTA. Values are mean sample absorbances interpolated against a calibration curve.

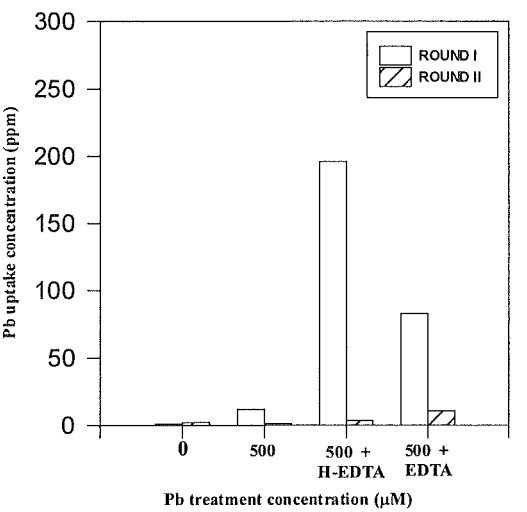


Figure 11b. Shoot Pb uptake from solution by seedlings of *Chamaecytisus palmensis* after 7 d in $Pb(NO_3)_2$ +/- 0.5 mM H-EDTA or EDTA. Values are mean sample absorbances interpolated against a calibration curve.

Table 10. Ratio of root Pb uptake to shoot Pb uptake for data depicted in figure 11

Treatment	Round 1	Round 2
0 Pb	4.37	0.74
500 µM Pb	21.32	69.18
500 µM Pb + H-EDTA	0.05	3.38
500 µM Pb + EDTA	0.05	0.81

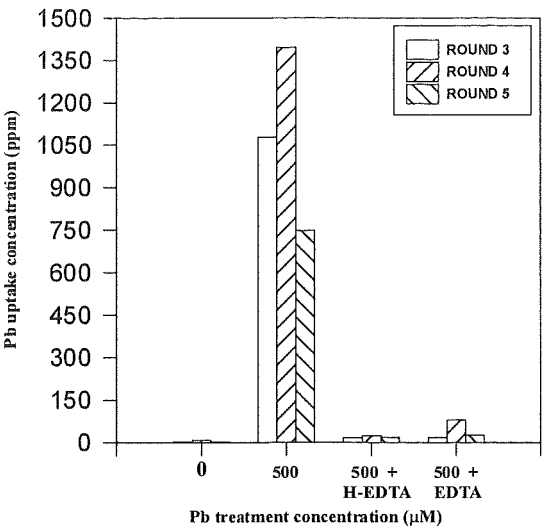


Figure 12a. Root Pb uptake from solution by seedlings of *Chamaecytisus palmensis* after 7 d in $Pb(NO_3)_2$ +/- 0.5 mM H-EDTA or EDTA. Values are mean sample absorbances interpolated against a calibration curve.

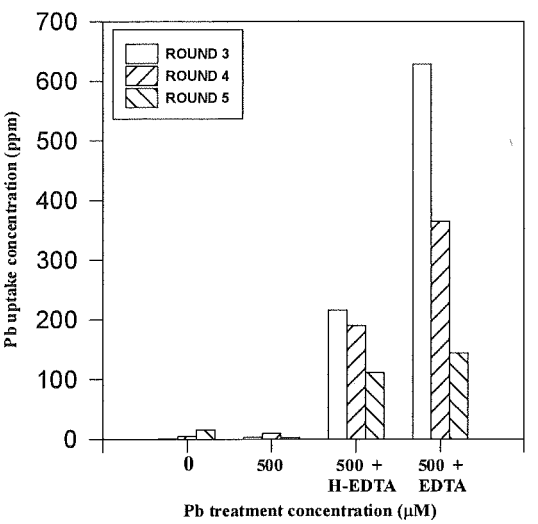


Figure 12b. Shoot Pb uptake from solution by seedlings of *Chamaecytisus palmensis* after 7 d in $Pb(NO_3)_2$ +/- 0.5 mM H-EDTA or EDTA. Values are mean sample absorbances interpolated against a calibration curve.

Table 11. Ratio of root Pb uptake to shoot Pb uptake for data depicted in figure 12

Treatment	Round 3	Round 4	Round 5
0 Pb	3.31	1.76	0.13
500 µM Pb	417.87	150.19	314.53
500 µM Pb + H-EDTA	0.07	0.12	0.16
500 µM Pb + EDTA	0.03	0.22	0.18

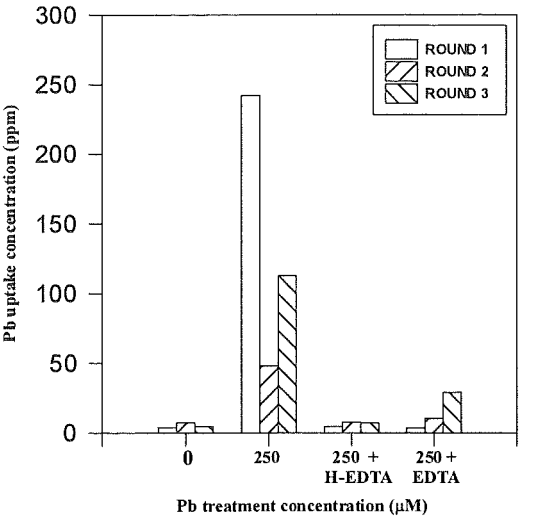


Figure 13a. Root Pb uptake from solution by seedlings of *Chamaecytisus palmensis* after 7 d in $Pb(NO_3)_2$ +/- 0.5 mM H-EDTA or EDTA. Values are mean sample absorbances interpolated against a calibration curve.

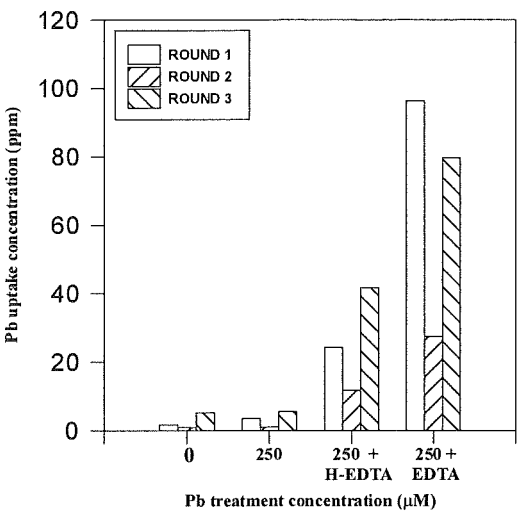


Figure 13b. Shoot Pb uptake from solution by seedlings of *Chamaecytisus palmensis* after 7 d in $Pb(NO_3)_2$ +/- 0.5 mM H-EDTA or EDTA. Values are mean sample absorbances interpolated against a calibration curve.

Table 12. Ratio of root Pb uptake to shoot Pb uptake for data depicted in figure 13

Treatment	Round 1	Round 2	Round 3
0 Pb	2.26	8.01	0.89
250 μM Pb	67.83	46.50	20.26
250 μM Pb + H-EDTA	0.19	0.66	0.17
250 μM Pb + EDTA	0.04	0.38	0.36

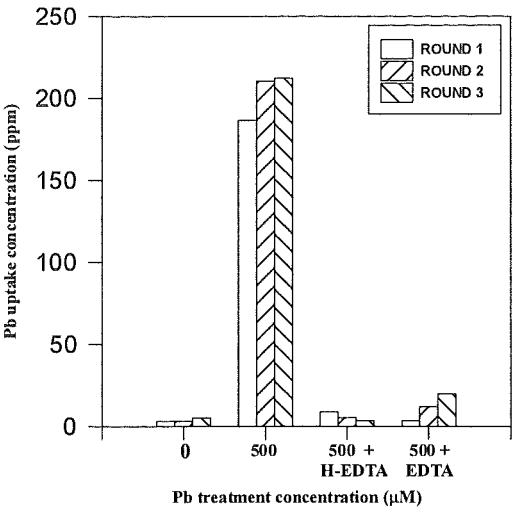


Figure 14a. Root Pb uptake from solution by seedlings of *Pinus radiata* after 7 d in $Pb(NO_3)_2$ +/- 0.5 mM H-EDTA or EDTA. Values are mean sample absorbances interpolated against a calibration curve.

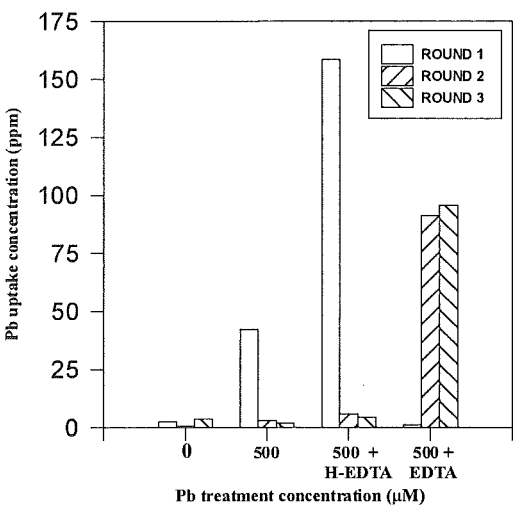


Figure 14b. Shoot Pb uptake from solution by seedlings of *Pinus radiata* after 7 d in $Pb(NO_3)_2$ +/- 0.5 mM H-EDTA or EDTA. Values are mean sample absorbances interpolated against a calibration curve.

Table 13. Ratio of root Pb uptake to shoot Pb uptake for data depicted in figure 14

Treatment	Round 1	Round 2	Round 3
0 Pb	1.21	5.08	1.44
500 μM Pb	4.42	70.10	117.20
500 μM Pb + H-EDTA	0.06	0.90	0.81
500 μM Pb + EDTA	3.12	0.13	0.21

At some treatment levels in these experiments less than 1 g (d.w.) shoot or root material was available for analysis at the conclusion. In all cases, calculated uptake levels were adjusted to 1g (d.w.) equivalent to preserve comparability of the samples.

Although variation was apparent in the magnitude of Pb uptake by both *C. palmensis* and *P. radiata* seedlings when considering all experimental rounds, certain trends were readily discernible. Unchelated Pb was taken up by roots to a much greater extent than shoots in every case, and chelated Pb was taken up by shoots to a greater extent than roots in almost every case. Typically the magnitude of Pb uptake by roots exceeded Pb uptake by shoots overall, and more often than not, shoot uptake of EDTA-chelated Pb exceeded shoot uptake of H-EDTA-chelated Pb. The level of uptake of Pb by *C. palmensis* at the 250 μM treatment level, with and without the addition of chelating agents, appeared to be lower than the level of Pb uptake at the 500 μM treatment level.

At the conclusion of these experiments adverse plant effects due to high chelator concentrations, particularly with H-EDTA, were still apparent on *C. palmensis* seedlings. In general, *P. radiata* seedlings were affected less by high chelator concentrations than *C. palmensis* seedlings but in 0.5 mM H-EDTA, pine needles tended to discolour slightly, turning brown along the axis, particularly at the apex of the plant. Also, whole *P. radiata* plants in 0.5 mM H-EDTA tended to be a lighter shade of green than those in other treatments, at the conclusion of the experiment.

(b) Aerated: When the treatment solutions were not aerated there was a tendency for them to become discoloured and malodorous, particularly with *C. palmensis*, by the time the experiment was terminated, due to the growth of microorganisms. Although these conditions did not seem to adversely affect the plants it was considered desirable to improve the treatment conditions so that effects due to Pb treatment, or chelation, would not be attributed to other causes. Subsequently, continuous aeration was incorporated into all experiments to prevent the growth of microorganisms (Plate 9A).

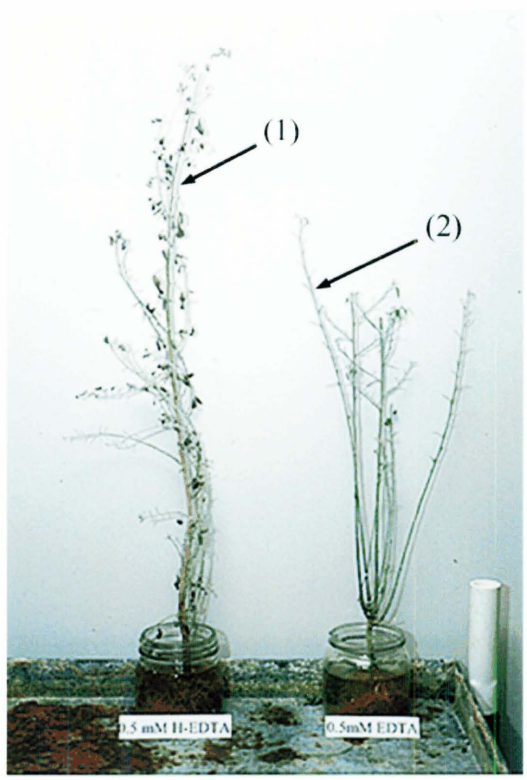
PLATE 9 Images depicting continuous aeration of *Chamaecytisus palmensis* in hydroponic culture, and adverse effects on foliage due to chelation treatment generally.

(A) Arrows indicate (1) compressed air supply and (2) offtake line supplying air to sparge stone in treatment vessel

(B) Arrows indicate (1) blackened, dehydrated foliage due to H-EDTA exposure, some of which remained attached and (2) almost complete leaf-blade drop due to EDTA exposure.

(C) Arrows indicate (1) intact shoot apex after H-EDTA treatment and (2) detail of petiole remaining attached to stem after leaf-blade detachment from pulvinus.

(A) Lead uptake experimental apparatus showing *Chamaecytisus palmensis* seedlings with continuous aeration.



(B) *Chamaecytisus palmensis* showing post-treatment effects of chelation by (left) 0.5 mM H-EDTA and (right) 0.5 mM EDTA.

(C) *Chamaecytisus palmensis* showing effects on foliage after 7 days in 500 µM Pb + 0.125 mM H-EDTA.

PLATE 9

After the introduction of continuous aeration, *C. palmensis* seedlings were exposed to 250 μM Pb, with and without chelation at 0.5 mM, for 1 round (Figure 15). At the conclusion of this, and all subsequent experiments, the treatment solutions were all clear and practically odourless, presumably due to aeration. Plants treated with chelators, particularly H-EDTA, still showed signs of stress including leaf drop and as a result of this, all subsequent exposure of *C. palmensis* to H-EDTA was at a reduced concentration of 0.125 mM.

C. palmensis seedlings were exposed to 500 μM Pb, with and without chelation at 0.125 mM, for 3 rounds (Figure 16). At the reduced chelator concentration (0.125 mM), plants exposed to H-EDTA in particular, were still exhibiting stress symptoms, mainly pronounced leaf drop, at the conclusion of the experiment (Plate 9C). *P. radiata* seedlings were exposed to 500 μM Pb, with and without chelation at 0.5 mM, for 3 rounds (Figure 17), and 250 μM Pb, with and without chelation at 0.5 mM, for 3 rounds (Figure 18).

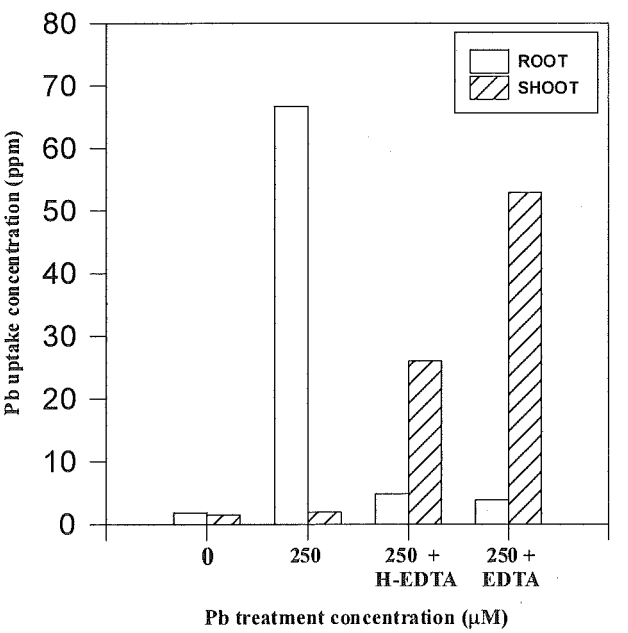


Figure 15. **Pb uptake from aerated solution by seedlings of *Chamaecytisus palmensis* after 7 d in $Pb(NO_3)_2$ +/- 0.5 mM H-EDTA or EDTA. Values are mean sample absorbances interpolated against a calibration curve.**

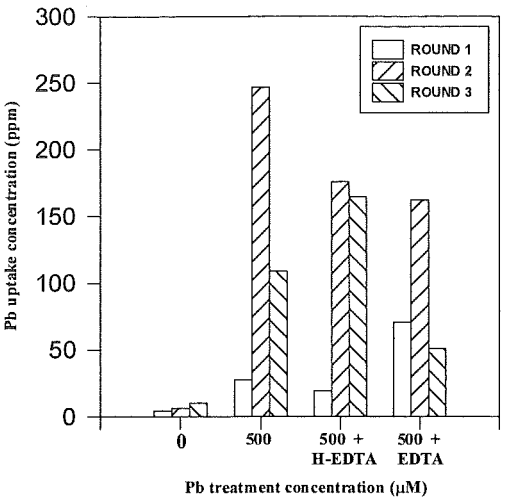


Figure 16a. Root Pb uptake from aerated solution by seedlings of *Chamaecytisus palmensis* after 7 d in $Pb(NO_3)_2$ +/- 0.125 mM H-EDTA or EDTA. Values are mean absorbances interpolated against a calibration curve.

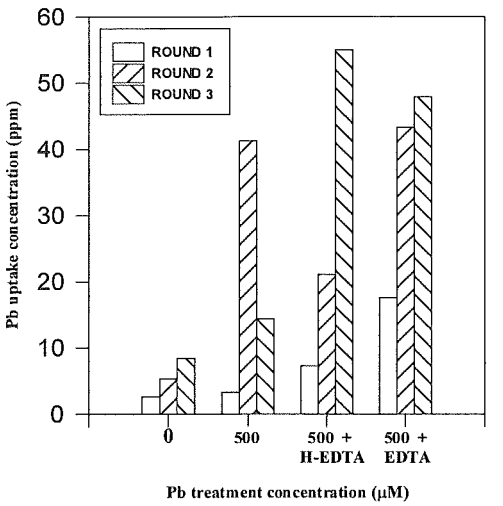


Figure 16b. Shoot Pb uptake from aerated solution by seedlings of *Chamaecytisus palmensis* after 7 d in $Pb(NO_3)_2$ +/- 0.125 mM H-EDTA or EDTA. Values are mean absorbances interpolated against a calibration curve.

Table 14. Ratio of root Pb uptake to shoot Pb uptake for data depicted in figure 16

Treatment	Round 1	Round 2	Round 3
0 Pb	1.69	1.19	1.21
500 µM Pb	8.45	5.98	7.61
500 µM Pb + H-EDTA	2.67	8.34	3.00
500 µM Pb + EDTA	4.02	3.74	1.07

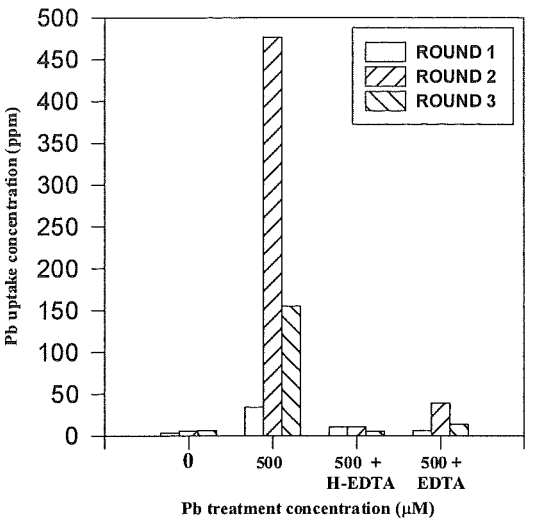


Figure 17a. Root Pb uptake from aerated solution by seedlings of *Pinus radiata* after 7 d in $Pb(NO_3)_2$ +/- 0.5 mM H-EDTA or EDTA. Values are mean sample absorbances interpolated against a calibration curve.

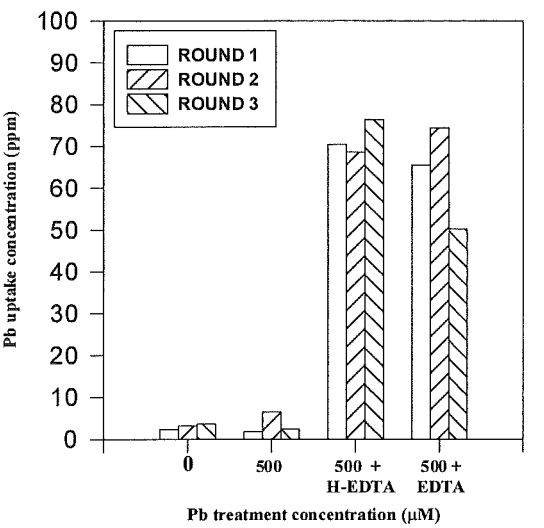


Figure 17b. Shoot Pb uptake from aerated solution by seedlings of *Pinus radiata* after 7 d in $Pb(NO_3)_2$ +/- 0.5 mM H-EDTA or EDTA. Values are mean sample absorbances interpolated against a calibration curve.

Table 15 Ratio of root Pb uptake to shoot Pb uptake for data depicted in figure 17

Treatment	Round 1	Round 2	Round 3
0 Pb	1.45	1.77	1.80
500 μM Pb	18.98	74.08	63.87
500 μM Pb + H-EDTA	0.15	0.16	0.07
500 μM Pb + EDTA	0.10	0.53	0.27

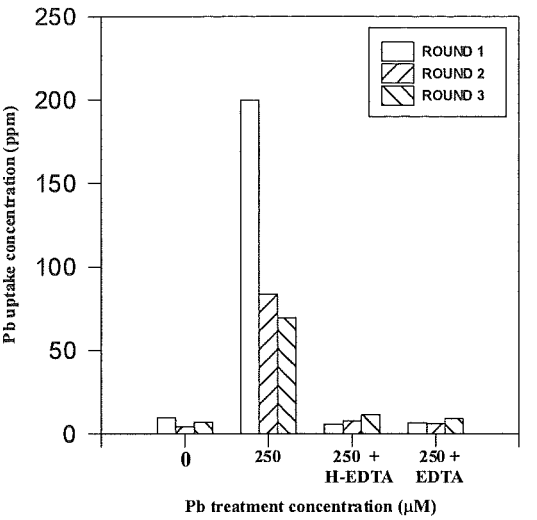


Figure 18a. Root Pb uptake from aerated solution by seedlings of *Pinus radiata* after 7 d in $Pb(NO_3)_2$ +/- 0.5 mM H-EDTA or EDTA. Values are mean sample absorbances interpolated against a calibration curve.

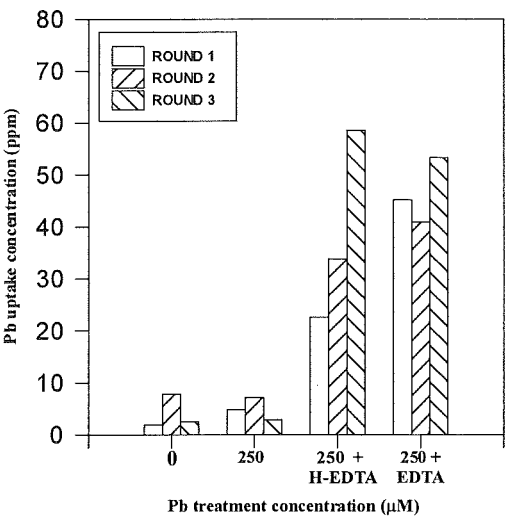


Figure 18b. Shoot Pb uptake from aerated solution by seedlings of *Pinus radiata* after 7 d in $Pb(NO_3)_2$ +/- 0.5 mM H-EDTA or EDTA. Values are mean sample absorbances interpolated against a calibration curve.

Table 16. Ratio of root Pb uptake to shoot Pb uptake for data depicted in figure 18

Treatment	Round 1	Round 2	Round 3
0 Pb	4.99	0.55	2.69
250 μM Pb	41.58	11.69	23.81
250 μM Pb + H-EDTA	0.25	0.23	0.20
250 μM Pb + EDTA	0.14	0.15	0.17

As in the uptake experiments conducted without aeration, variation in the magnitude of Pb uptake levels was encountered in the uptake experiments conducted with aeration. Definite trends though, are apparent in the pattern of Pb uptake and deposition. Generally, the same conclusions hold true: roots take up mainly unchelated Pb, and shoots take up mainly chelated Pb. However, some anomalous results are depicted in Figure 16, where *C. palmensis* roots have taken up significant amounts of chelated Pb, and *C. palmensis* shoots have taken up small amounts of unchelated Pb. This was an unusual result, not typical of the majority of experiments conducted.

In the case of *P. radiata,* there seems to be some evidence for a concentration-dependent effect on uptake. At the 500 μM Pb treatment level, uptake by roots of unchelated Pb was nearly twice the uptake at the 250 μM Pb treatment level. It is not clear whether shoot uptake of chelated Pb is enhanced more by H-EDTA or EDTA for *P. radiata* seedlings. Shoot uptake of chelated Pb by *C. palmensis* seedlings seems to be enhanced more by EDTA than H-EDTA.

4.2.5 Pb uptake by clones grown in solution

P. radiata clone S was exposed to 500 μM Pb plus chelator at 0.5 mM for 2 rounds (Figure 19). *P. radiata* clones T, V, W, and X were exposed to 500 μM Pb plus chelator at 0.5 mM (Figure 20). *C. palmensis* clone 7 was exposed to 500 μM Pb, plus or minus H-EDTA at 0.125 mM, or EDTA at 0.5 mM, for 3 rounds (Figure 21). *C. palmensis* clones 1, 2, and 5 were exposed to 500 μM Pb plus or minus H-EDTA at 0.125 mM, or EDTA at 0.5 mM (Figure 22). At 0.125 mM H-EDTA, extensive leaf drop was evident on the treated plants at the conclusion of the experiments. These leaves were collected and analysed together with the plants they originated on. Shoot tips on these plants remained viable. Pb uptake patterns by *P. radiata* clones followed the trends initially displayed by *P. radiata* seedlings. Roots took up significantly more unchelated Pb than shoots, shoots took up more chelated Pb than roots, and overall, roots took up more Pb than shoots.

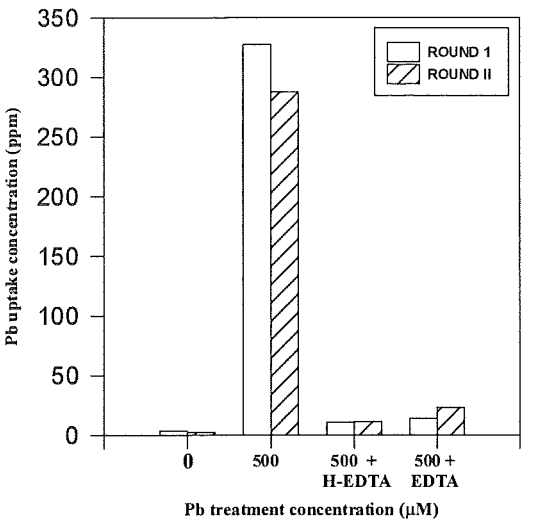


Figure 19a. Root Pb uptake from aerated solution by *Pinus radiata* clone 'S' after 7 d in $Pb(NO_3)_2$ +/- 0.5 mM H-EDTA or EDTA. Values are mean sample absorbances interpolated against a calibration curve.

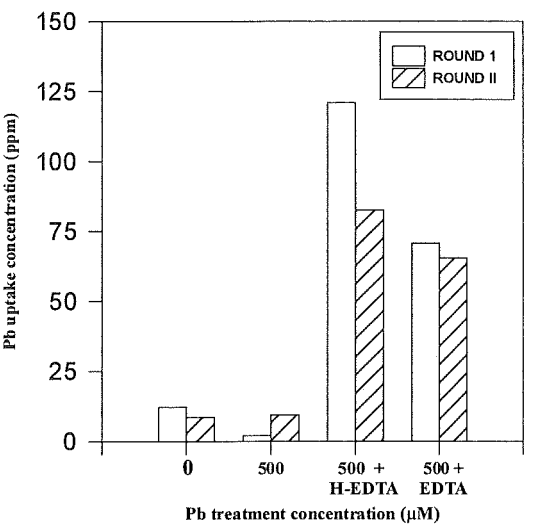


Figure 19b. Shoot Pb uptake from aerated solution by *Pinus radiata* clone 'S' after 7 d in $Pb(NO_3)_2$ +/- 0.5 mM H-EDTA or EDTA. Values are mean sample absorbances interpolated against a calibration curve.

Table 17. Ratio of root Pb uptake to shoot Pb uptake for data depicted in figure 19

Treatment	Round 1	Round 2
0 Pb	0.28	0.30
500 µM Pb	162.94	30.59
500 µM Pb + H-EDTA	0.09	0.14
500 µM Pb + EDTA	0.20	0.36

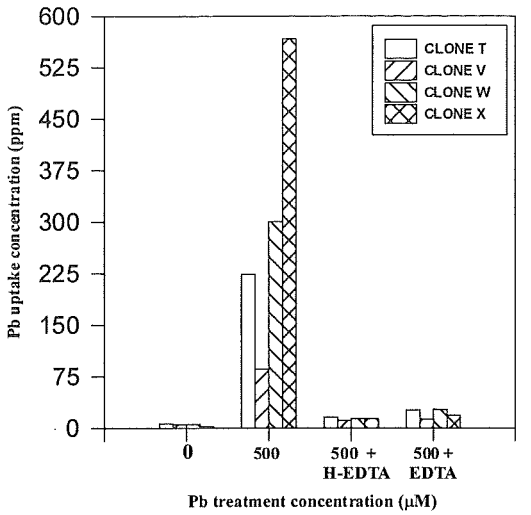


Figure 20a. Root Pb uptake from aerated solution by *Pinus radiata* clones T, V, W, and X after 7 d in $Pb(NO_3)_2$ +/- 0.5 mM H-EDTA or EDTA. Values are mean absorbances interpolated against a calibration curve.

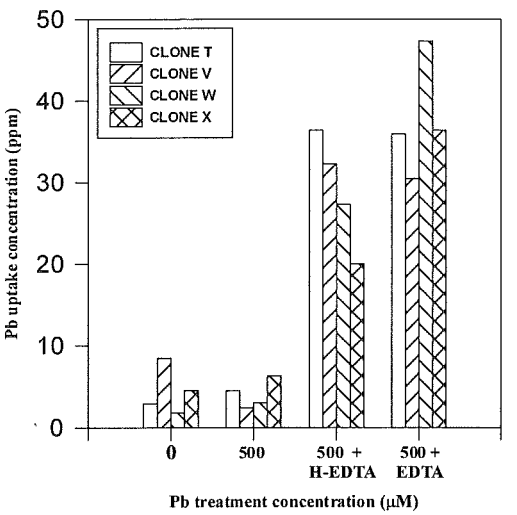


Figure 20b. Shoot Pb uptake from aerated solution by *Pinus radiata* clones T, V, W, and X after 7 d in $Pb(NO_3)_2$ +/- 0.5 mM H-EDTA or EDTA. Values are mean absorbances interpolated against a calibration curve.

Table 18. Ratio of root Pb uptake to shoot Pb uptake for data depicted in figure 20

Treatment	Clone T	Clone V	Clone W	Clone X
0 Pb	2.27	0.60	3.06	0.46
500 μM Pb	50.06	35.35	100.55	89.98
500 μM Pb + H-EDTA	0.44	0.34	0.50	0.66
500 μM Pb + EDTA	0.73	0.42	0.58	0.51

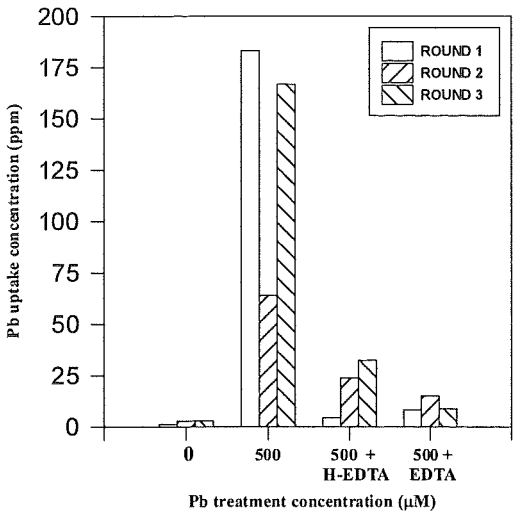


Figure 21a. Root Pb uptake from aerated solution by *Chamaecytisus palmensis* clone 7 after 7 d in $Pb(NO_3)_2$ +/- 0.125 mM H-EDTA or 0.5 mM EDTA. Values are mean absorbances interpolated against a calibration curve.

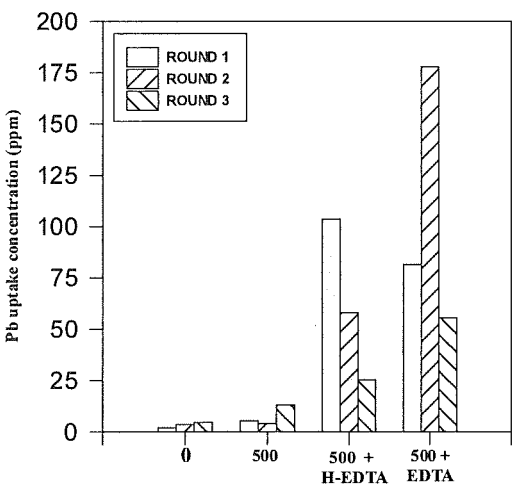


Figure 21b. Shoot Pb uptake from aerated solution by *Chamaecytisus palmensis* clone 7 after 7 d in $Pb(NO_3)_2$ +/- 0.125 mM H-EDTA or 0.5 mM EDTA. Values are mean absorbances interpolated against a calibration curve.

Table 19 Ratio of root Pb uptake to shoot Pb uptake for data depicted in figure 21

Treatment	Round 1	Round 2	Round 3
0 Pb	0.69	0.80	0.64
500 μM Pb	34.25	16.48	12.80
500 μM Pb + H-EDTA	0.04	0.41	1.28
500 μM Pb + EDTA	0.10	0.09	0.16

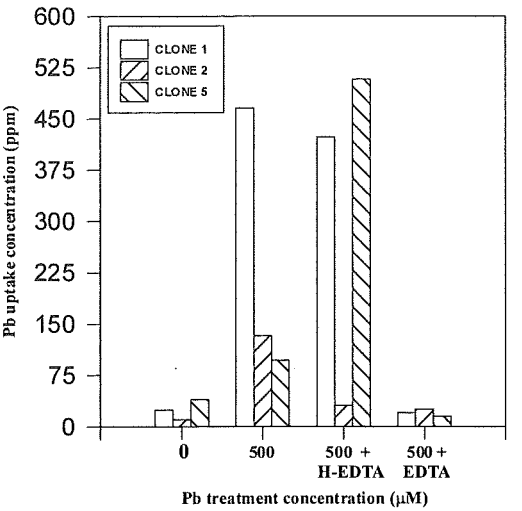


Figure 22a. Root Pb uptake from aerated solution by *Chamaecytisus palmensis* clones 1, 2, and 5 after 7 d in $Pb(NO_3)_2$ +/- 0.125 mM H-EDTA or 0.5 mM EDTA. Values are means interpolated against a calibration curve.

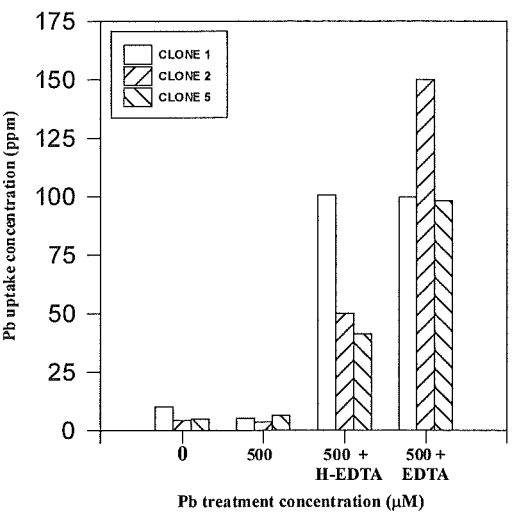


Figure 22b. Shoot Pb uptake from aerated solution by *Chamaecytisus palmensis* clones 1, 2, and 5 after 7 d in $Pb(NO_3)_2$ +/- 0.125 mM H-EDTA or 0.5 mM EDTA. Values are means interpolated against a calibration curve.

Table 20 Ratio of root Pb uptake to shoot Pb uptake for data depicted in figure 22

Treatment	Clone 1	Clone 2	Clone 5
0 Pb	2.40	2.34	8.17
500 μM Pb	87.82	36.74	15.13
500 μM Pb + H-EDTA	4.20	0.62	12.32
500 μM Pb + EDTA	0.20	0.17	0.15

The same is essentially true for the *C. palmensis* clones, particularly #7 and to a lesser degree #2. Clones #1 and #5 displayed unusually high root uptake of H-EDTA chelated Pb but shoot uptake patterns were consistent with the majority of other experimental results.

4.2.6 Pb uptake data:analysis of variance

The P values for the factor 'treatment' from all the analyses are presented in table 21. For factor 'round', only two P values with significance at the 99 % level were found. These were (1) figure 11 & 12 root, and (2) figure 13 shoot. For factor 'clone' no significant P values were found. Full anova tables are presented in appendix E. Unplanned multiple comparisons of means were carried out using the Tukey test with standard error for equal sample sizes, on all Pb uptake data where anova found the treatment effect to be significant (Appendix F).

TABLE 21. P values for factor 'treatment', derived from 2 factor anova on Pb uptake data ($\alpha = 0.05$). Figures in italics are significant at the 99% level. Figures in bold type are significant at the 99.9% level.

	SPECIES	ROOT	SHOOT	ROOT/SHOOT
Figure 11 & 12	*C. palmensis*	**1.35×10^{-7}**	0.40667	**3.22×10^{-5}**
Figure 13	*C. palmensis*	*0.00871*	**1.874×10^{-5}**	**0.00098**
Figure 14	*P. radiata*	**0.000424**	0.517106	0.046515
Figure 16	*C. palmensis*	*0.006272*	0.019608	0.02755
Figure 17	*P. radiata*	*0.003697*	**3.03×10^{-5}**	**3.61×10^{-5}**
Figure 18	*P. radiata*	**0.000455**	*0.001093*	**0.000113**
Figure 19	*P. radiata*	*0.001134*	0.041072	*0.008692*
Figure 20	*P. radiata*	**8.54×10^{-6}**	**6.34×10^{-5}**	**6.2×10^{-7}**
Figure 21	*C. palmensis*	*0.00179*	*0.002514*	*0.003025*
Figure 22	*C. palmensis*	0.027705	**7.98×10^{-5}**	*0.003086*

(5) TRANSMISSION ELECTRON MICROSCOPY

All plant tissues analysed by transmission electron microscopy were subjected to Pb-uptake treatment for 7 days prior to E.M. specimen preparation. When examining ultra-thin sections in the T.E.M, with a view to selecting those that were the most representative of the effects of different levels of treatment, two major considerations were apparent. First was the level and location of any Pb deposition within the tissues due to treatment, and secondly, was whether or not the treatment itself had led to disruption or modification of normal cellular morphology. For these purposes, the inclusion and interpretation of non-Pb treated controls was essential. A number of stained *Pinus radiata* root sections were included to illustrate both the general effects of staining on cellular components, and also to determine if any normal cellular entities might possibly, after staining, resemble Pb deposits, and therefore mask the effects of treatment. In general, unstained sections showed adequate detail. For each tissue type, the control (non-Pb treated) micrographs are presented first to provide a basis for comparison.

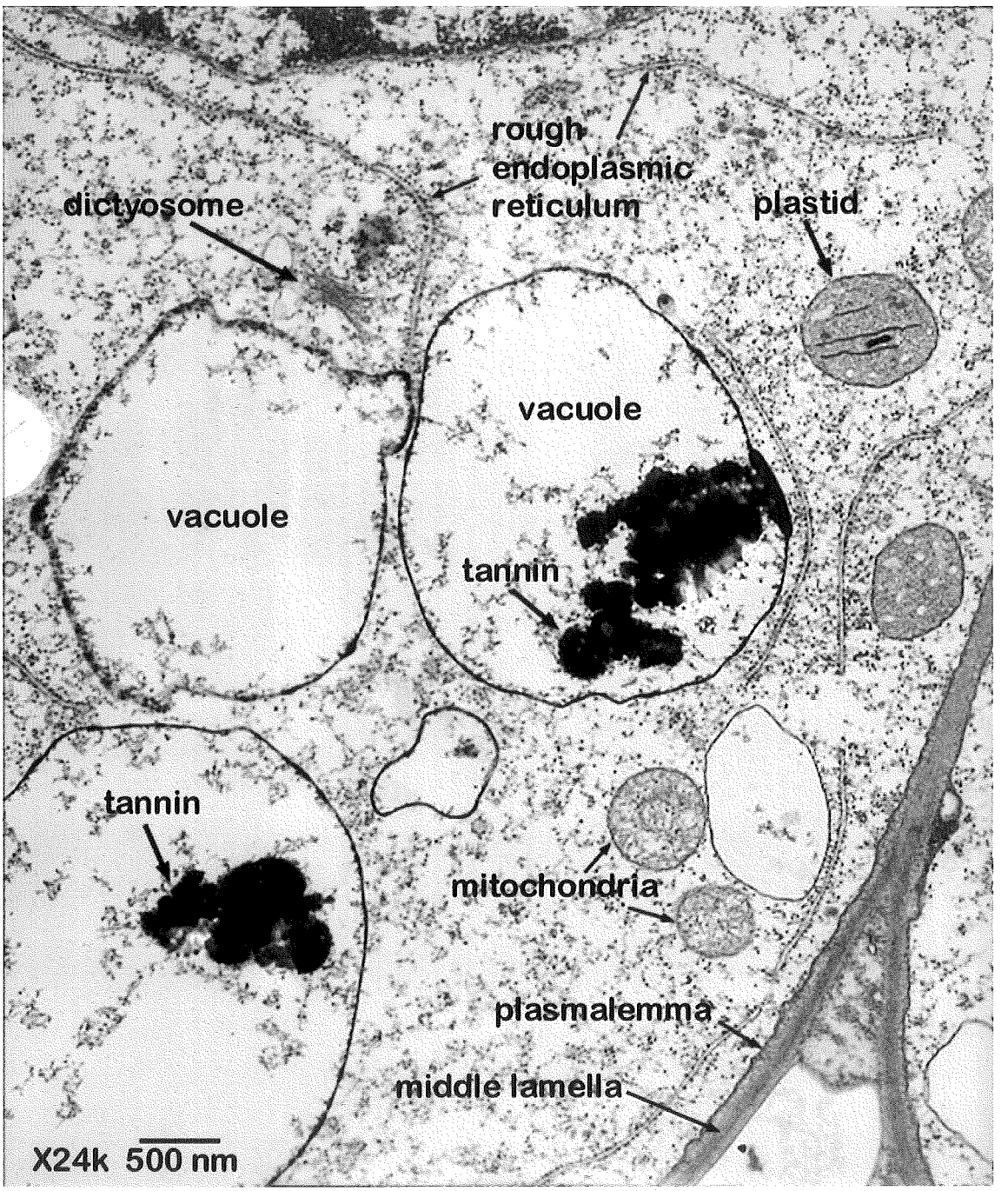
5.1 Electron microscopic observations

5.1.1 P. radiata

Stained sections of non-Pb treated P. radiata root tissue (Plates 10 & 11).

Among the many cellular components evident in Plate 10, at a magnification of 24,000X, are several vacuoles. The small size of the vacuoles indicates that these cells were not fully mature. Some of the vacuoles contain large, electron-dense, globular aggregates of tannin. Ribosomes can clearly be seen as dark, bead-like structures attached to the endoplasmic reticulum, and also free in the cytoplasm (Plate 10). In the nucleus, darkly-stained nucleoplasm is evident among lighter patchy regions, at 20,000X magnification (Plate 11). In both plates the composite nature of the cell wall can clearly be seen.

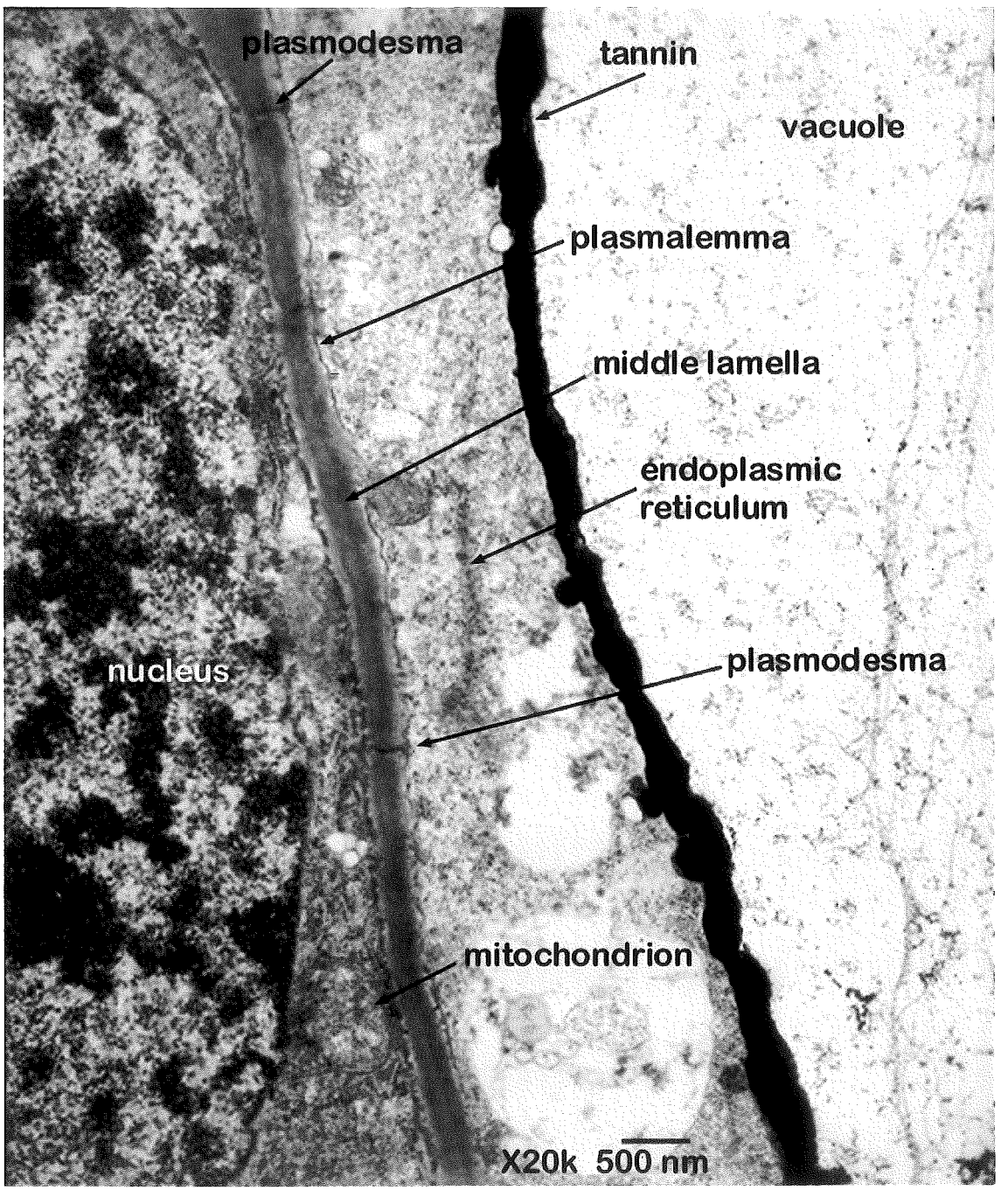
PLATE 10 Transmission electron micrograph of stained ultra-thin section of non-Pb treated *Pinus radiata* root at 24,000 X magnification.



Pinus radiata root O Pb (stained)

PLATE 10

PLATE 11 Transmission electron micrograph of stained ultra-thin section of non-Pb treated *Pinus radiata* root at 20,000 X magnification.



Pinus radiata root 0 Pb (stained)

PLATE 11

Stained and unstained sections of P. radiata root tissue exposed to 500 μM Pb (Plates 12-14).

Within a stained section (Plate 12a), at 15,000X magnification, comprising the intersection of several moderately vacuolated cells surrounding an intercellular space, several large Pb particles can be seen closely associated with the cell wall, with the plasmalemma accommodating them in each case. In close-up view of the same section, at 150,000X magnification, it is possible to distinguish the plasmodesma that traverses the cell wall at the point at which the Pb particle is located (Plate 12b). The Pb particle itself has an irregular outline and is completely electron opaque. An unstained section, at 30,000X magnification, features the junction of two cells with several large Pb particles embedded in the cell wall (Plate 13a). In a nearby vacuole a tannin deposit can be seen. At 60,000X magnification, the jagged, irregular edge of the Pb grains can be seen clearly, as can the adjacent plasmodesma (Plate 13b). The morphology of cellular components, such as mitochondria, dictyosomes, ribosomes, and endoplasmic reticula, does not appear to have been adversely affected by the presence of Pb. Within an unstained section, at 24,000X magnification, a more substantial region of cell wall is evident with numerous, small Pb grains embedded in it (Plate 14a). The Pb grains are all associated with the outermost layer of the cell wall, adjacent to the plasmalemma. Nearby, a plastid containing an electron-dense plastoglobulus can be seen. At 100,000X magnification, the granular nature of the Pb particles is highlighted (Plate 14b).

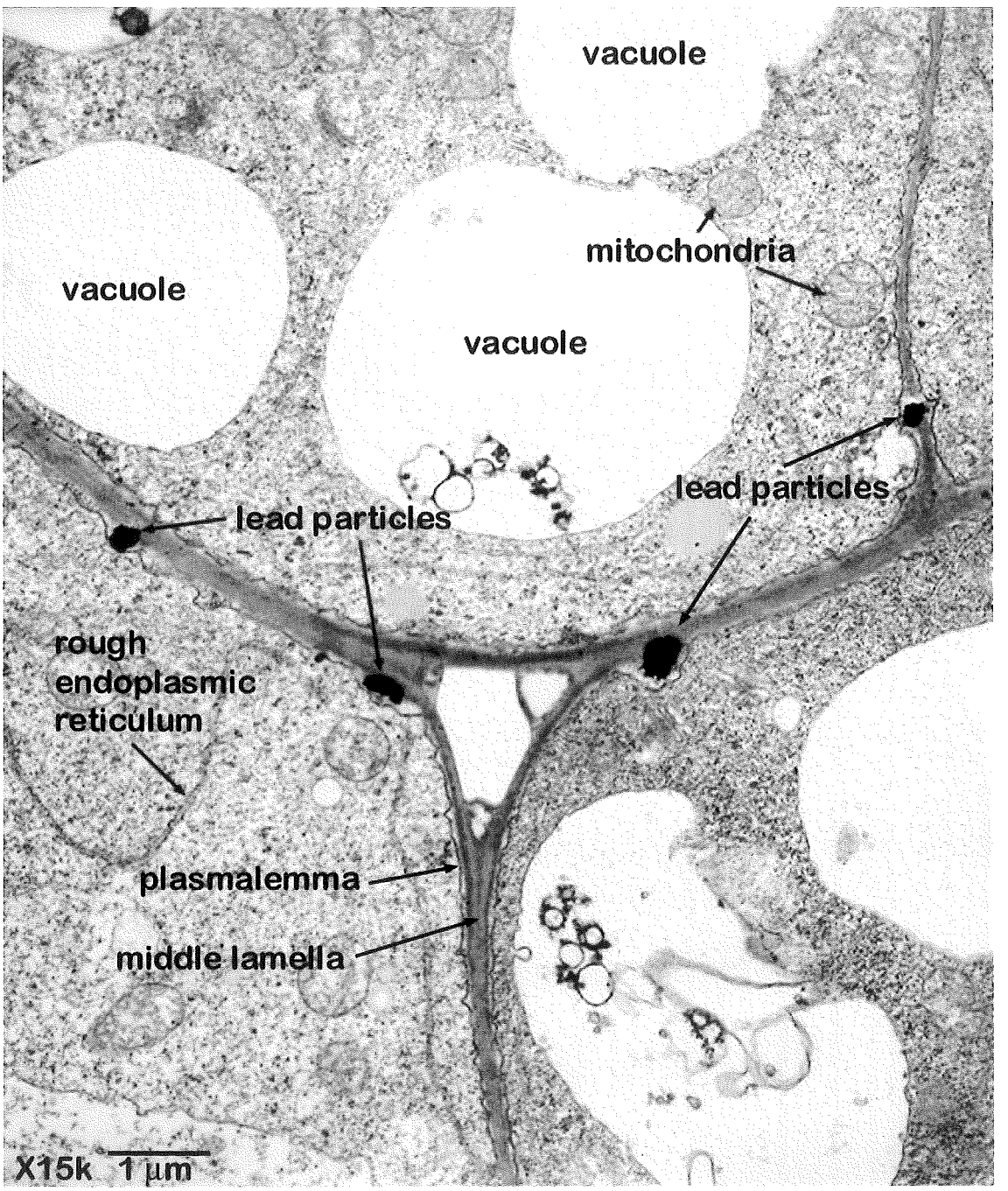
Stained section of P. radiata root tissue exposed to 500 μM Pb plus 0.5 mM H-EDTA (Plate 15).

At 20,000X magnification, a layer of tannin is seen associated with a large vacuole suggesting that these cells were more mature (Plate 15). Despite the very dark staining of the cell wall it was still possible to determine that no large Pb grains were associated with it and close examination of the surrounding regions did not detect deposition of finely dispersed Pb particles.

PLATES 12a & 12b Transmission electron micrographs of stained ultra-thin section of *Pinus radiata* root treated with 500 μM Pb for 7 days.

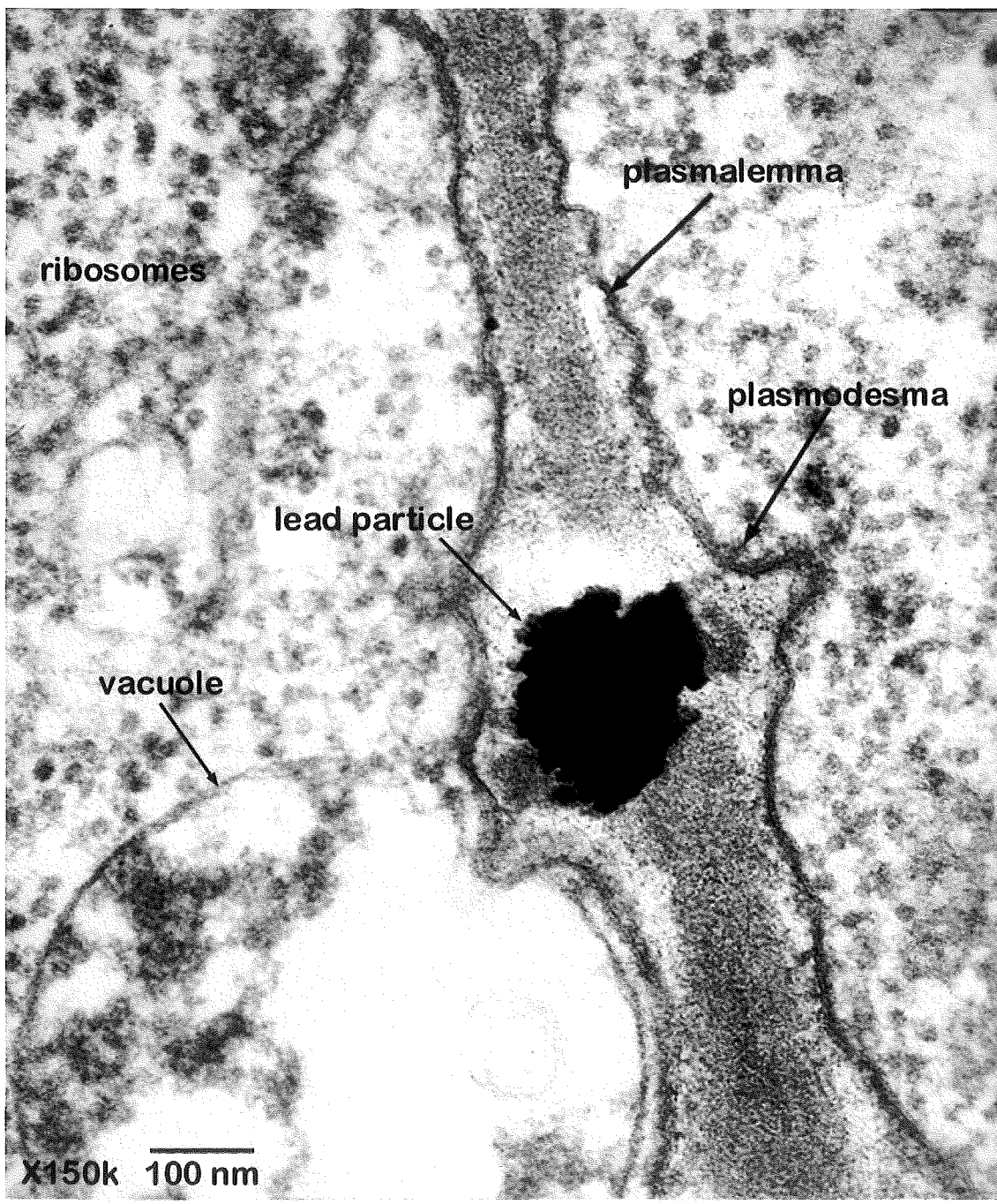
(12a) 15,000 X magnification

(12b) 150,000 X magnification



Pinus radiata root 500 µM Pb (stained)

PLATE 12a



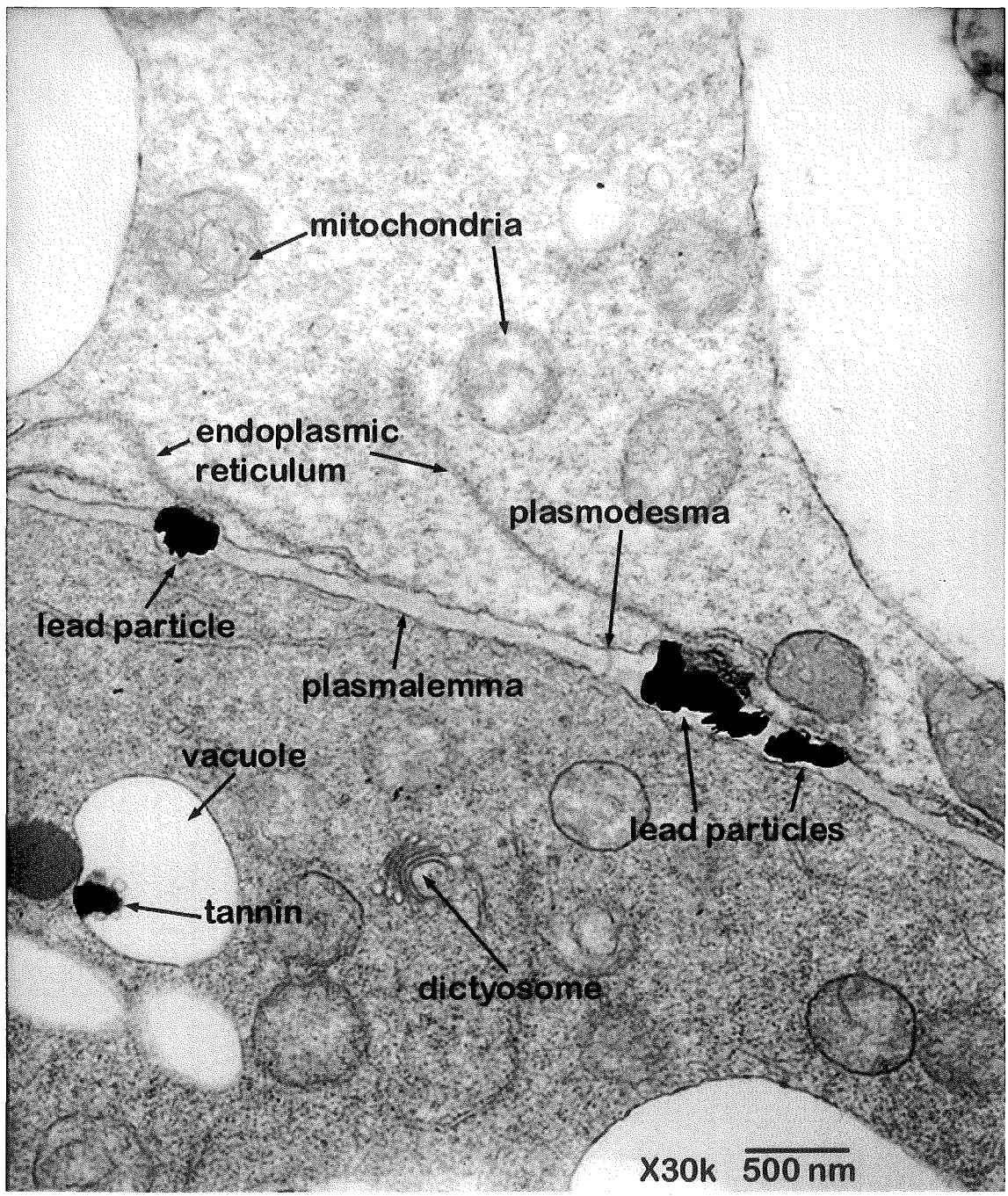
Pinus radiata root 500 µM Pb (stained)

PLATE 12b

PLATES 13a & 13b Transmission electron micrographs of ultra-thin section of *Pinus radiata* root treated with 500 μM Pb for 7 days.

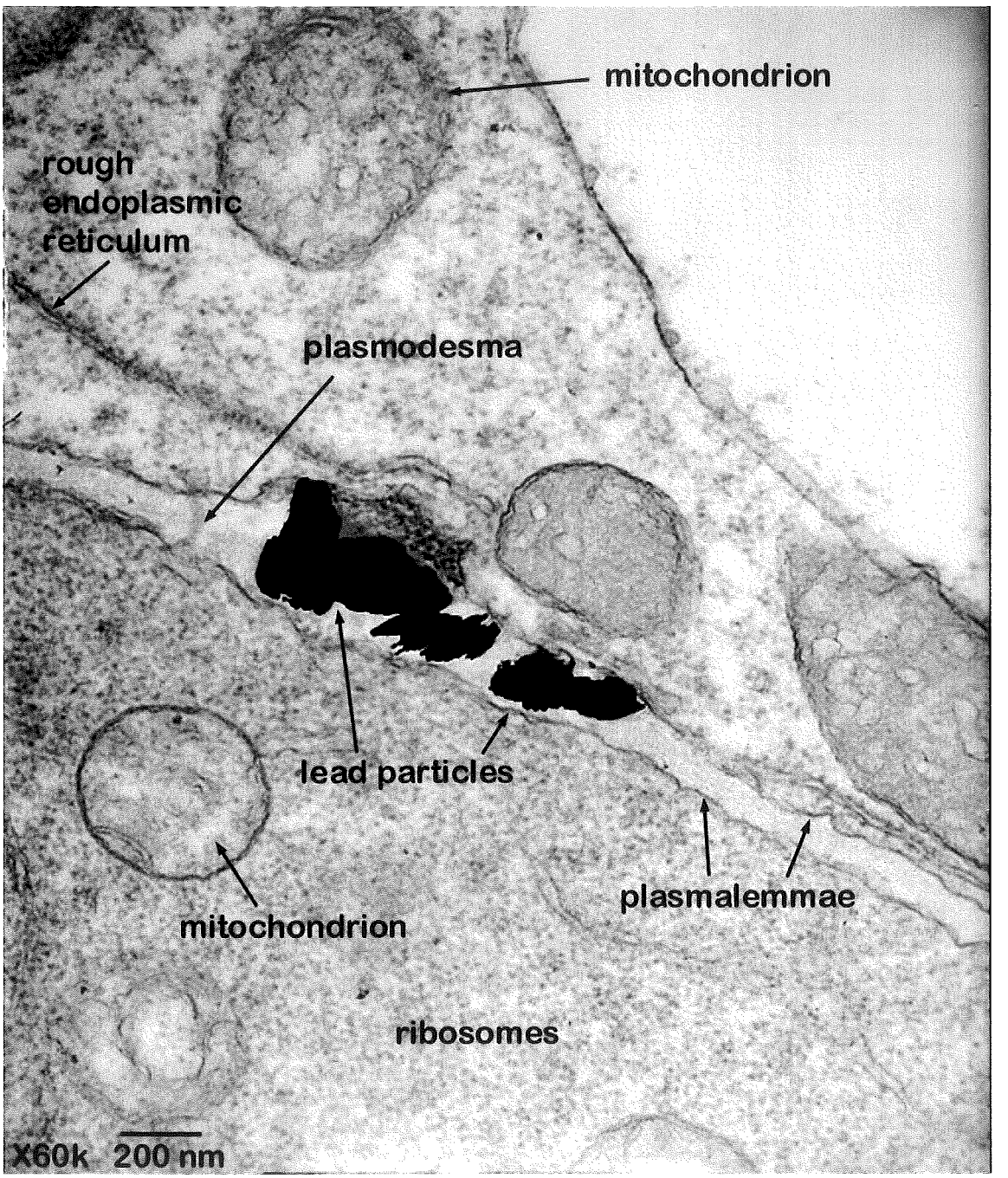
(13a) 30,000 X magnification

(13b) 60,000 X magnification



Pinus radiata root 500 µM Pb

PLATE 13a



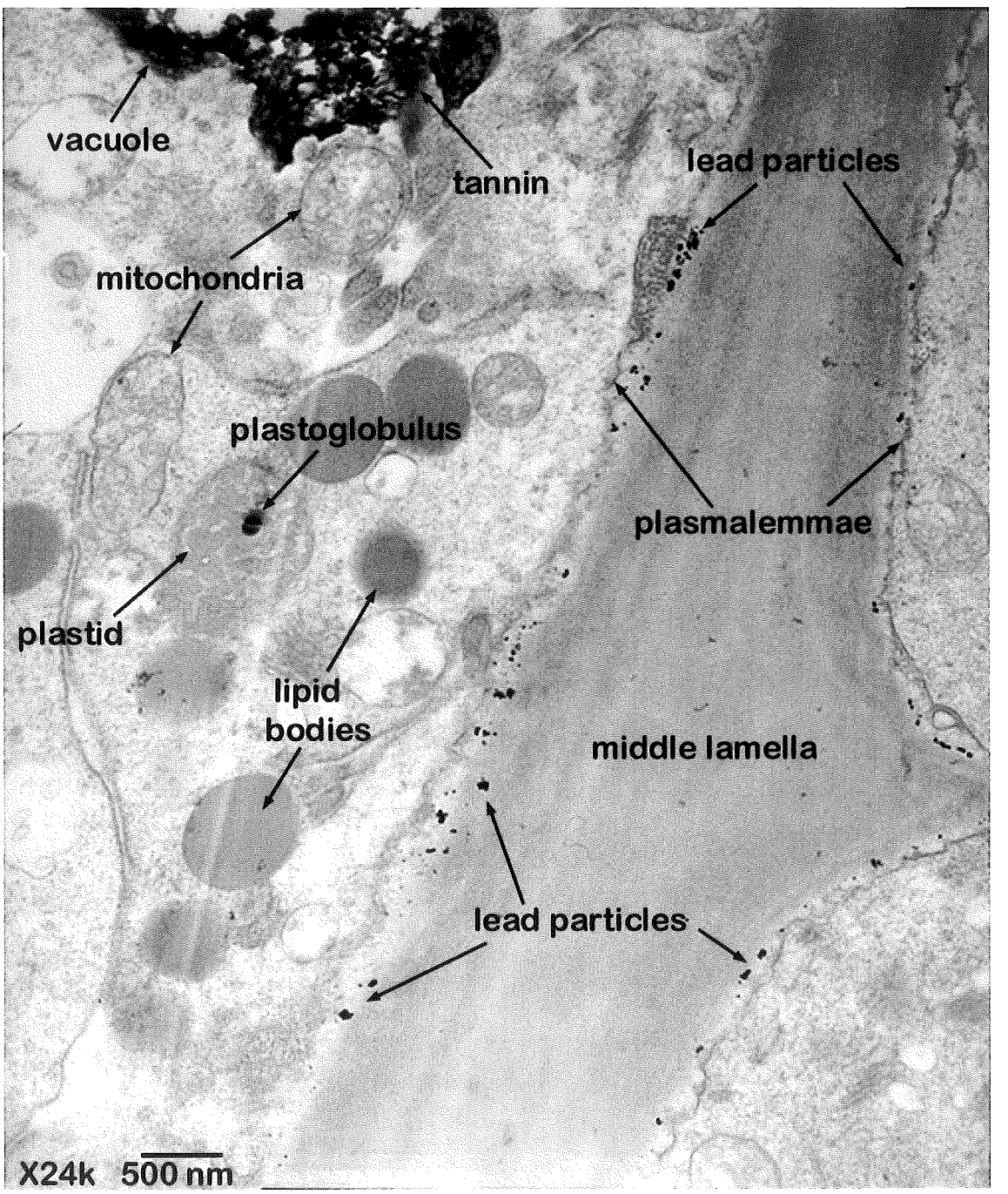
Pinus radiata root 500 µM Pb

PLATE 13b

PLATES 14a & 14b Transmission electron micrographs of ultra-thin section of *Pinus radiata* root treated with 500 μM Pb for 7 days.

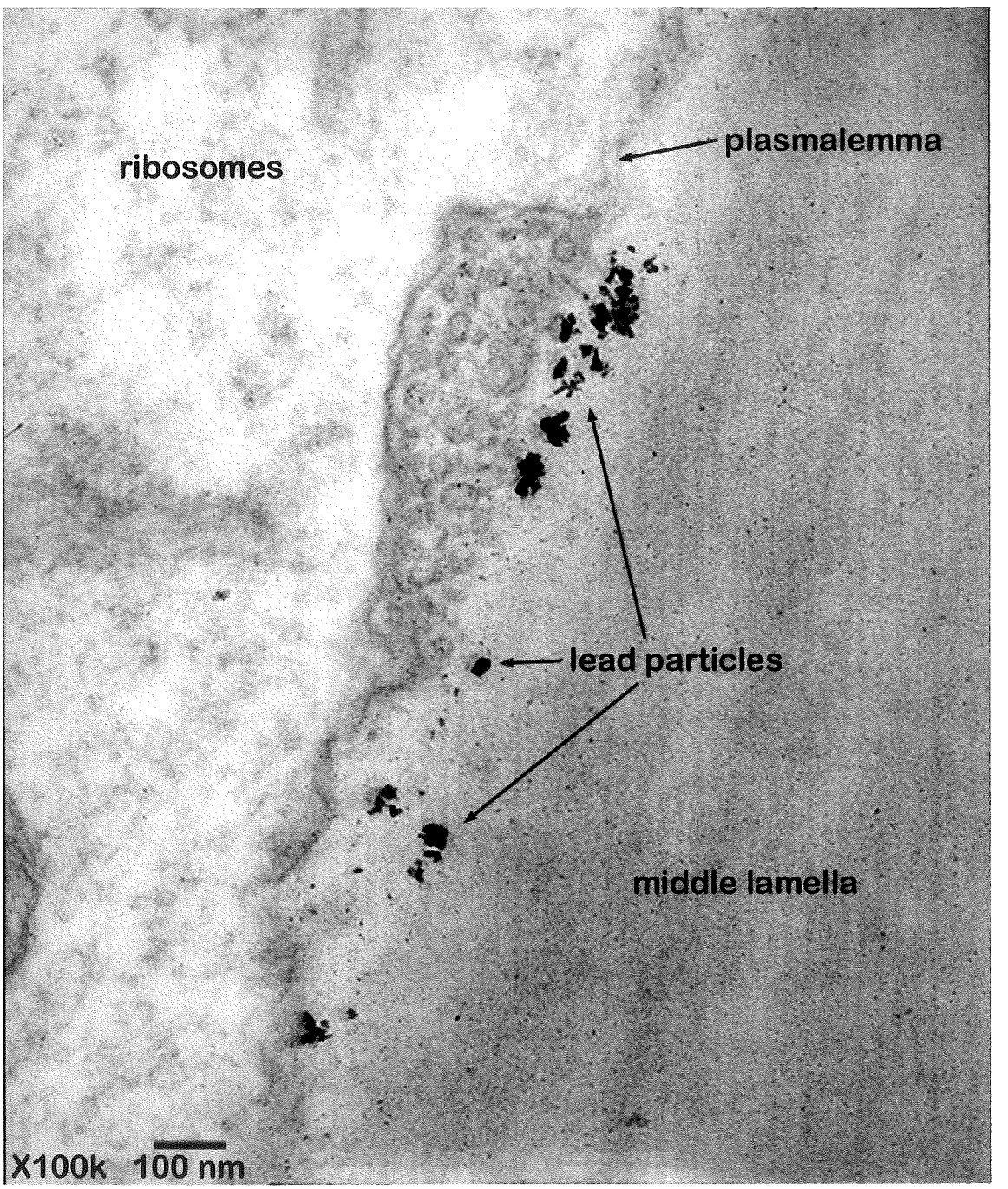
(14a) 24,000 X magnification

(14b) 100,000 X magnification



Pinus radiata root 500 µM Pb

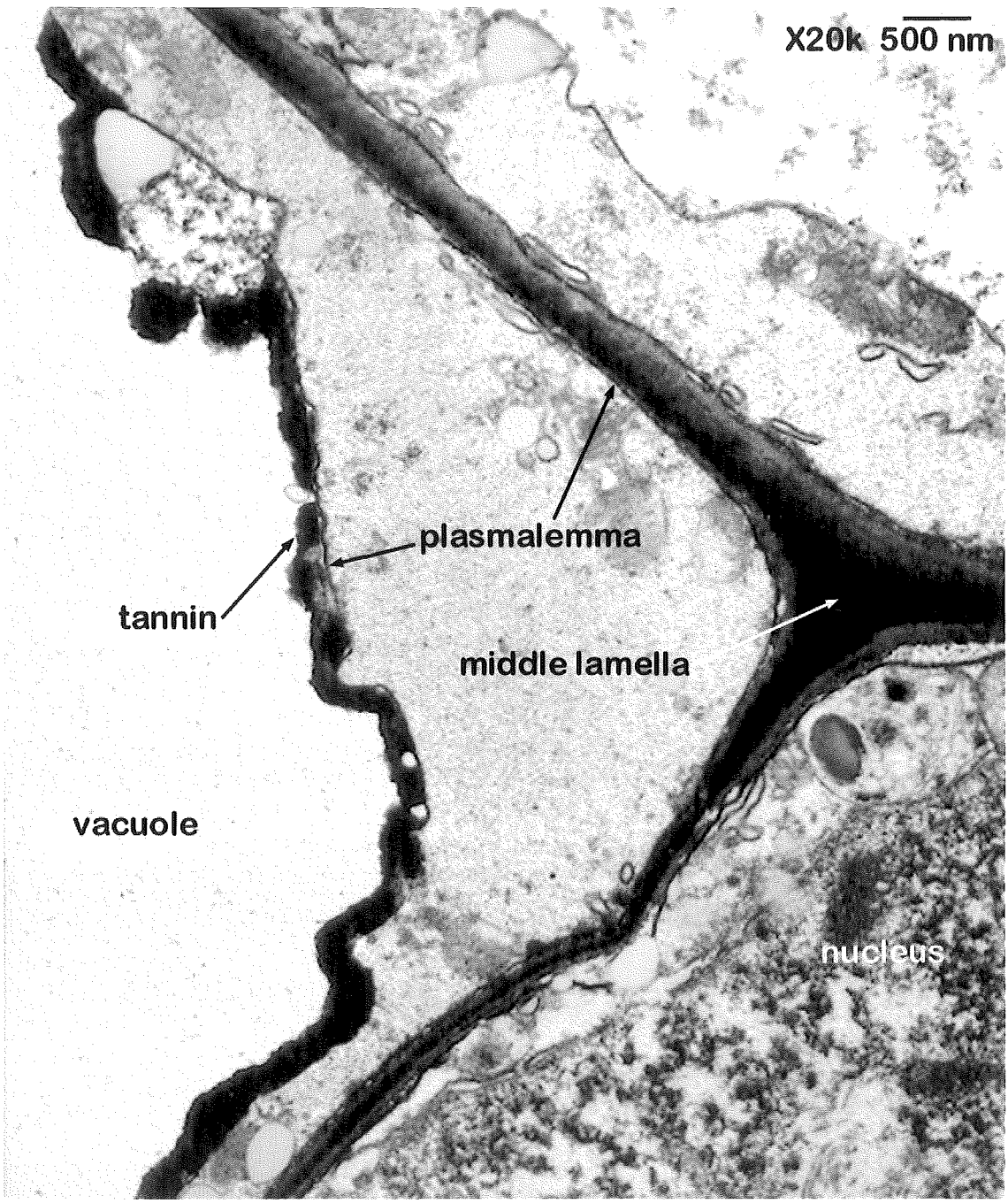
PLATE 14a



Pinus radiata root 500 µM Pb

PLATE 14b

PLATE 15 Transmission electron micrograph at 20,000 X magnification of stained ultra-thin section of *Pinus radiata* root treated with 500 μM Pb plus 0.5 mM H-EDTA, for 7 days.



Pinus radiata root 500 µM Pb 0.5 mM H-EDTA (stained)

PLATE 15

Unstained section of P. radiata root tissue exposed to 500 μM Pb plus 0.5 mM EDTA (Plate 16).

At 8,000X magnification, these cells appeared to be highly vacuolated and therefore quite mature, which explained the lack of organelles and cytoplasmic constituents (Plate 16). No large Pb grains were detected anywhere in the cell walls, including plasmodesmata, and examination of the surrounding tissues at higher magnification did not find evidence of finely dispersed Pb grains either.

Unstained section of non-Pb treated P. radiata shoot tissue (Plate 17).

At 15,000X magnification, these cells appeared highly vacuolated, fully mature, and were possibly xylem vessels (Plate 17). A thin layer of tannin can be seen at the junction of the plasmalemma and the outermost layer of the cell wall.

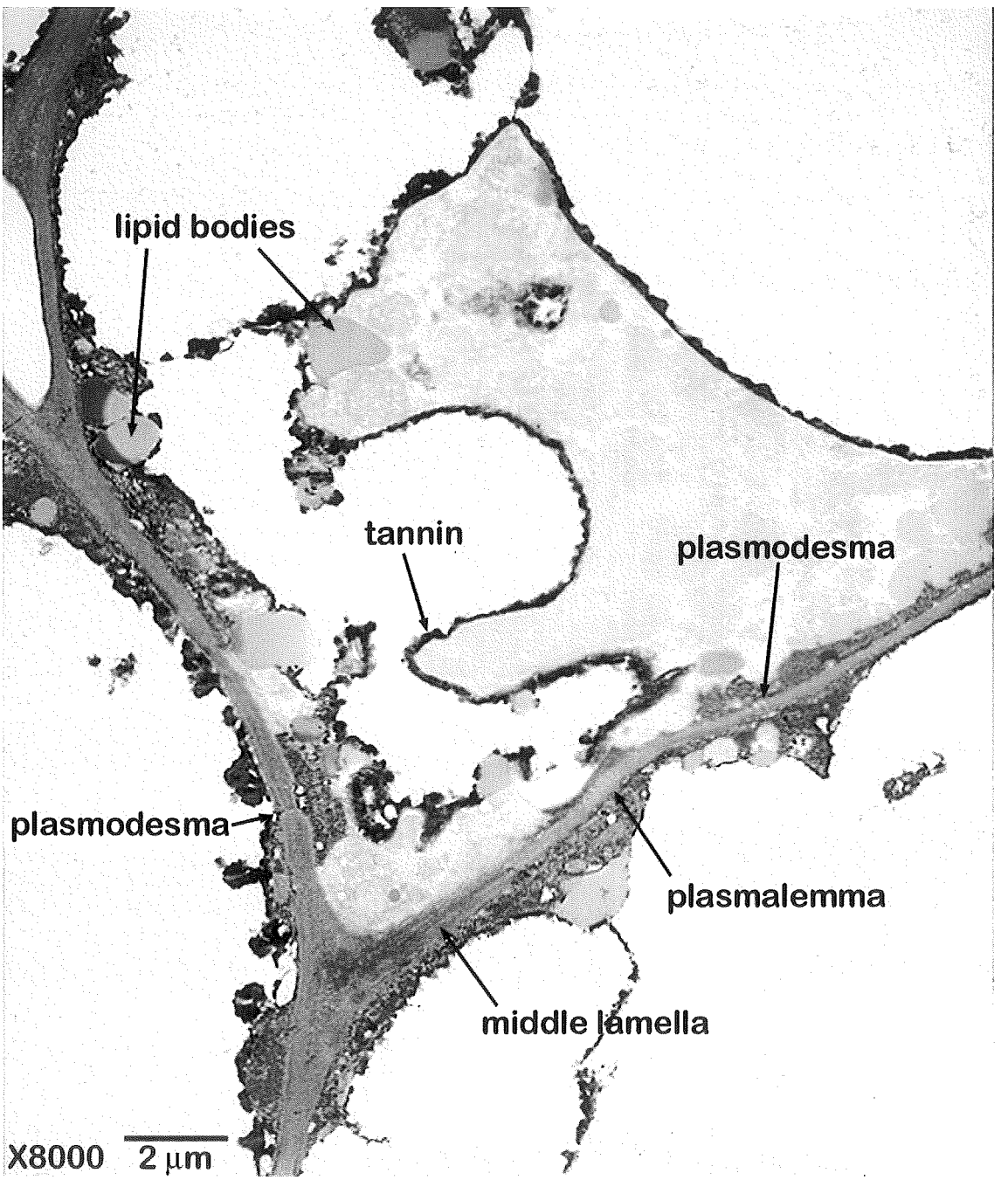
Unstained section of P. radiata shoot tissue treated with 500 μM Pb (Plate 18).

At 15,000X magnification, these cells appeared highly vacuolated, fully mature, and were possibly xylem vessels (Plate 18). Tannin deposits were in evidence both as continuous layers and as discrete clumps. Pb particles were not detected as either larger grains, such as those typically seen in root accumulation of unchelated Pb, or as finely dispersed particles.

Unstained section of P. radiata shoot tissue treated with 500 μM Pb plus 0.5 mM H-EDTA (Plate19).

At 120,000X magnification, Pb particles can be seen in the regions surrounding the cell wall (Plate 19). In this situation the Pb grains were only detectable at high magnification and were not found embedded in the cell wall but in the material adjacent to it. The object labeled as an artefact was not considered to be Pb due to its large size and smooth surface. In transmission electron microscopy artefacts such as this are seen from time to time and usually little importance is attached to them. In this case it was closer examination of the artefact that led to the discovery of the very finely dispersed Pb grains.

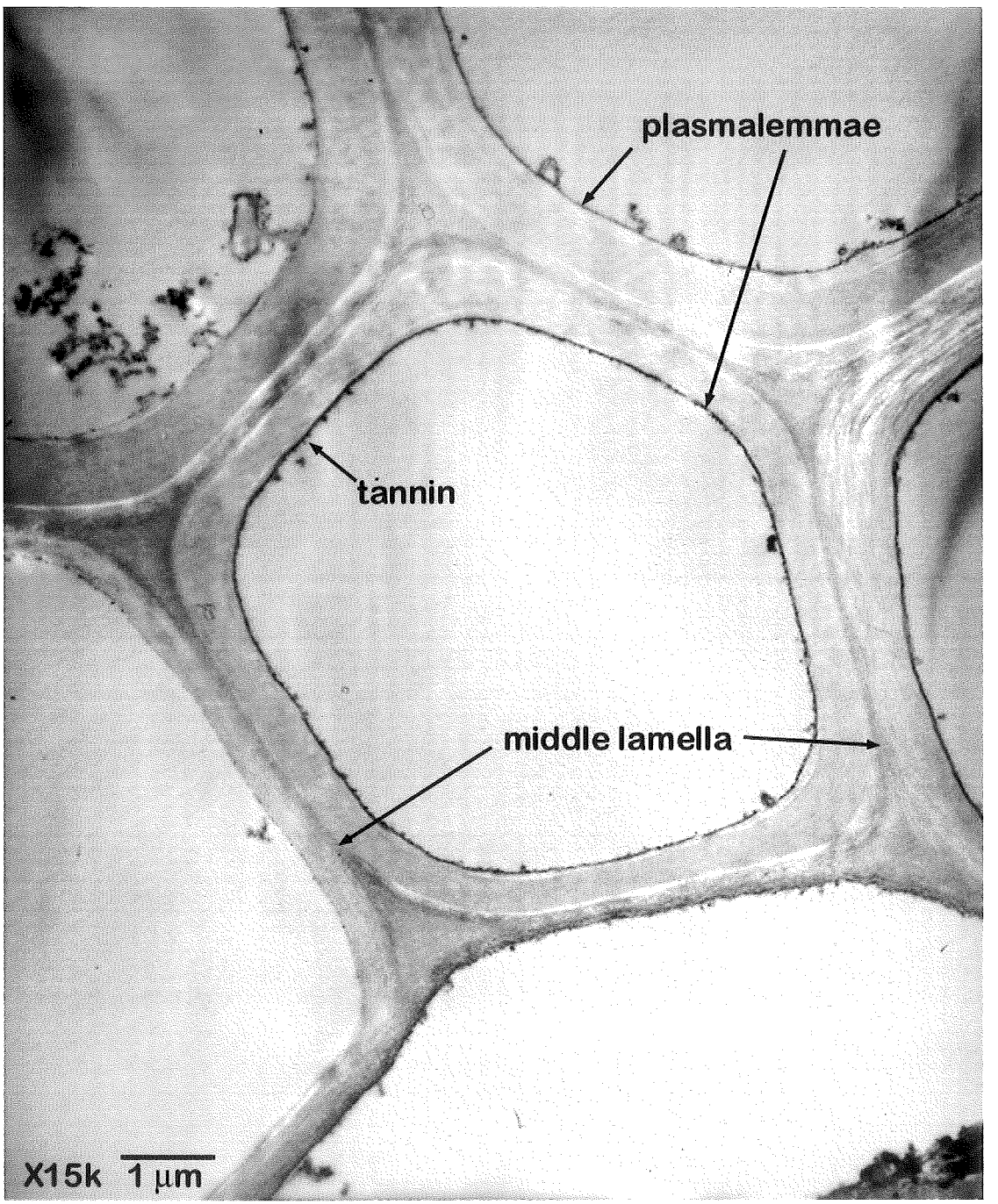
PLATE 16 Transmission electron micrograph at 8,000 X magnification of ultra-thin section of *Pinus radiata* root treated with 500 μM Pb plus 0.5 mM EDTA, for 7 days.



Pinus radiata root 500 µM Pb 0.5 mM EDTA

PLATE 16

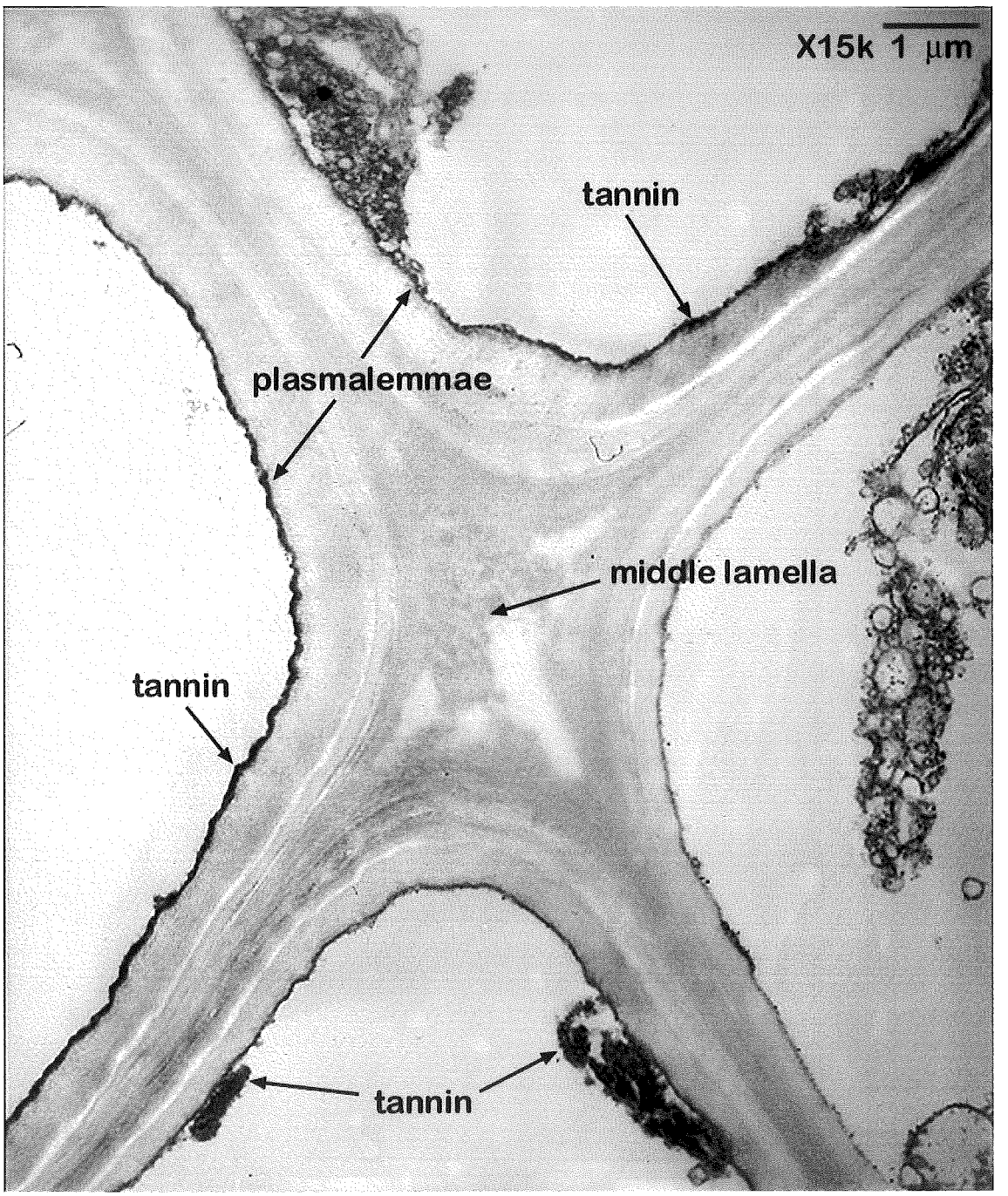
PLATE 17 Transmission electron micrograph of ultra-thin section of *Pinus radiata* non-Pb treated shoot at 15,000 X magnification.



Pinus radiata shoot 0 Pb

PLATE 17

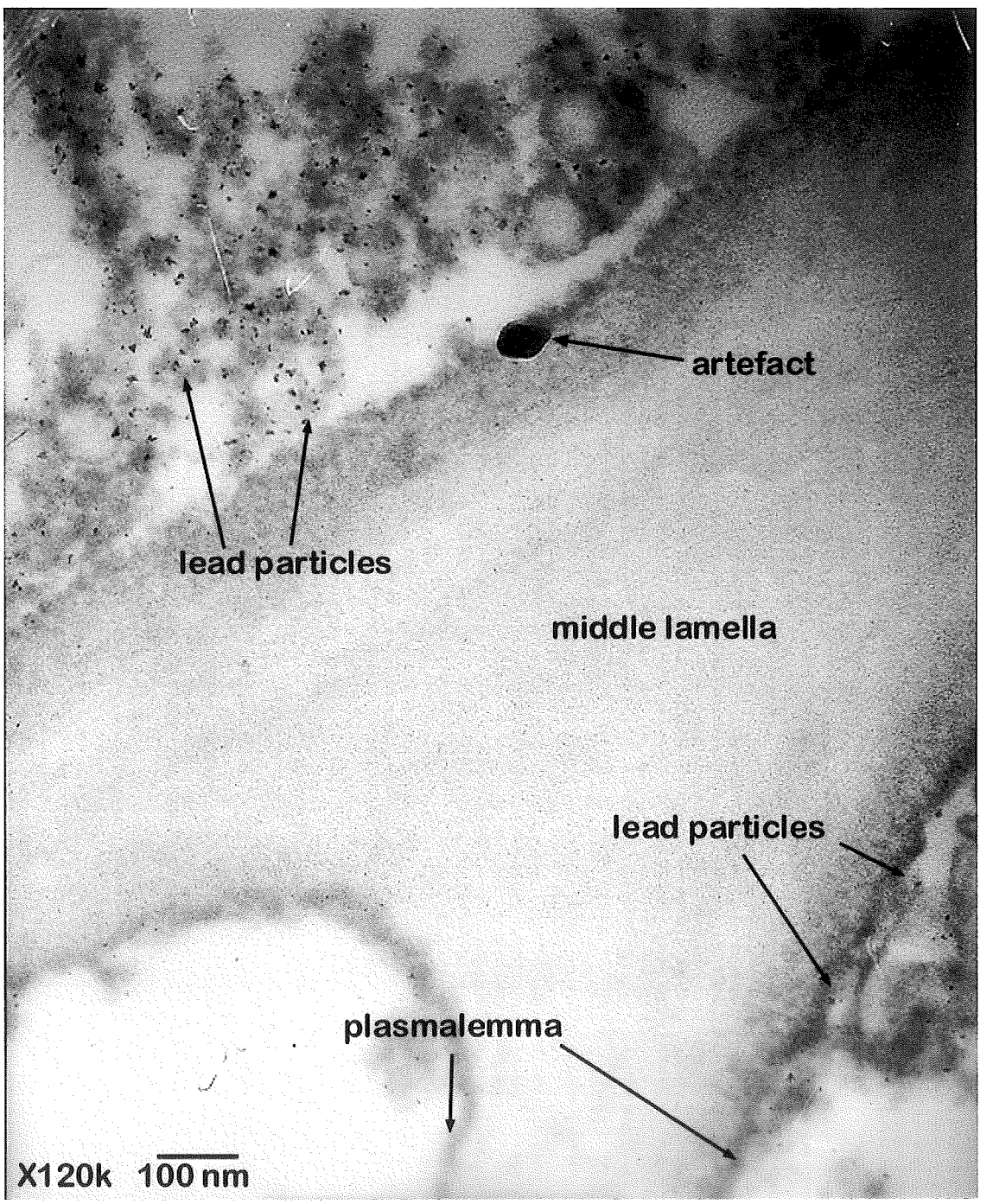
PLATE 18 Transmission electron micrograph at 15,000 X magnification of ultra-thin section of *Pinus radiata* shoot treated with 500 μM Pb, for 7 days.



Pinus radiata shoot 500 µM Pb

PLATE 18

PLATE 19 Transmission electron micrograph at 120,000 X magnification of ultra-thin section of *Pinus radiata* shoot treated with 500 μM Pb plus 0.5 mM H-EDTA, for 7 days.



Pinus radiata shoot 500 µM Pb 0.5 mM H-EDTA

PLATE 19

Unstained section of P. radiata shoot treated with 500 μM Pb plus 0.5 mM EDTA (Plate 20).

At 40,000X magnification, these cells appeared highly vacuolated and therefore probably quite mature (Plate 20). Heavy deposits of Pb were found in the intercellular space associated with these cells and little Pb was found in the cell wall itself, a phenomenon that was observed on several occasions.

5.1.2 C. palmensis

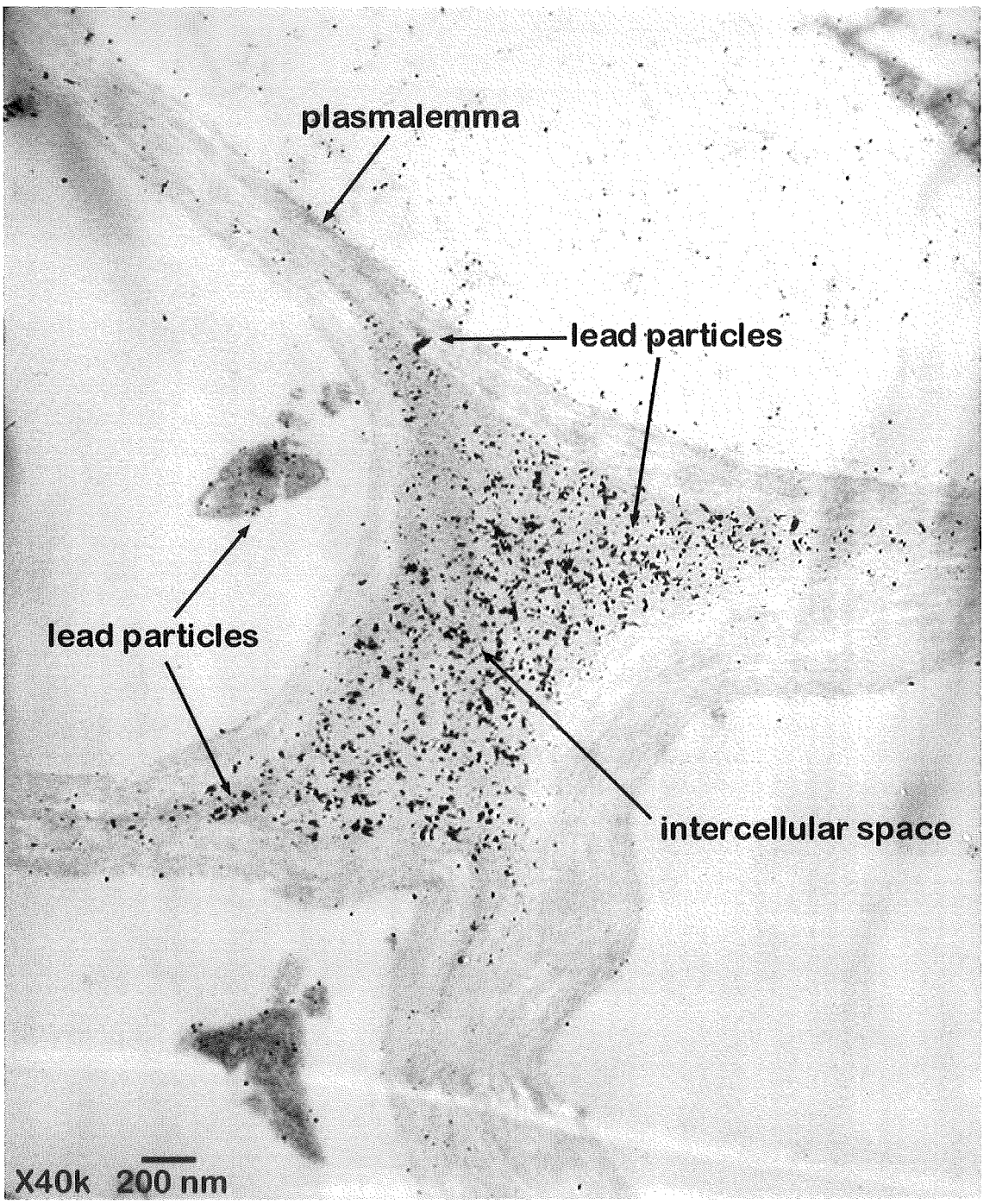
Section of a non-Pb treated C. palmensis root nodule (Plate 21).

At 24,000X magnification, numerous bacteroids were found in most cells, together with large numbers of mitochondria, a typical situation found in root nodule cells (Plate 21).

Sections of C. palmensis root nodules treated with 500 μM Pb (Plates 22 & 23).

Four cells can be seen surrounding an intercellular space, at 15,000X magnification (Plate 22a). Heavy deposits of small Pb particles were found in bacteroids, mitochondria, and cell walls, particularly those enclosing the intercellular space, where some very large Pb particles were also discovered. In some bacteroids, Pb particles are seen distributed randomly throughout their structure, while in others heavy Pb deposits are concentrated in their outer membranes. In some sections of the cell wall, particularly those surrounding the intercellular space, the Pb deposits appear to have completely occupied the volume of the cell wall. At 40,000X magnification, the magnitude of the Pb deposition, particularly around the intercellular space but also in the mitochondrion and the outer layers of the bacteroids, can be seen in detail (Plate 22b). A region of the root nodule where the cells are bounded by a layer of endodermis, can be seen at 60,000X magnification (Plate 23). The root nodule endodermis is thought to function as a diffusion layer separating regions of different osmolarity, restricting the exchange of gases and nutrients (Vincent, 1974).

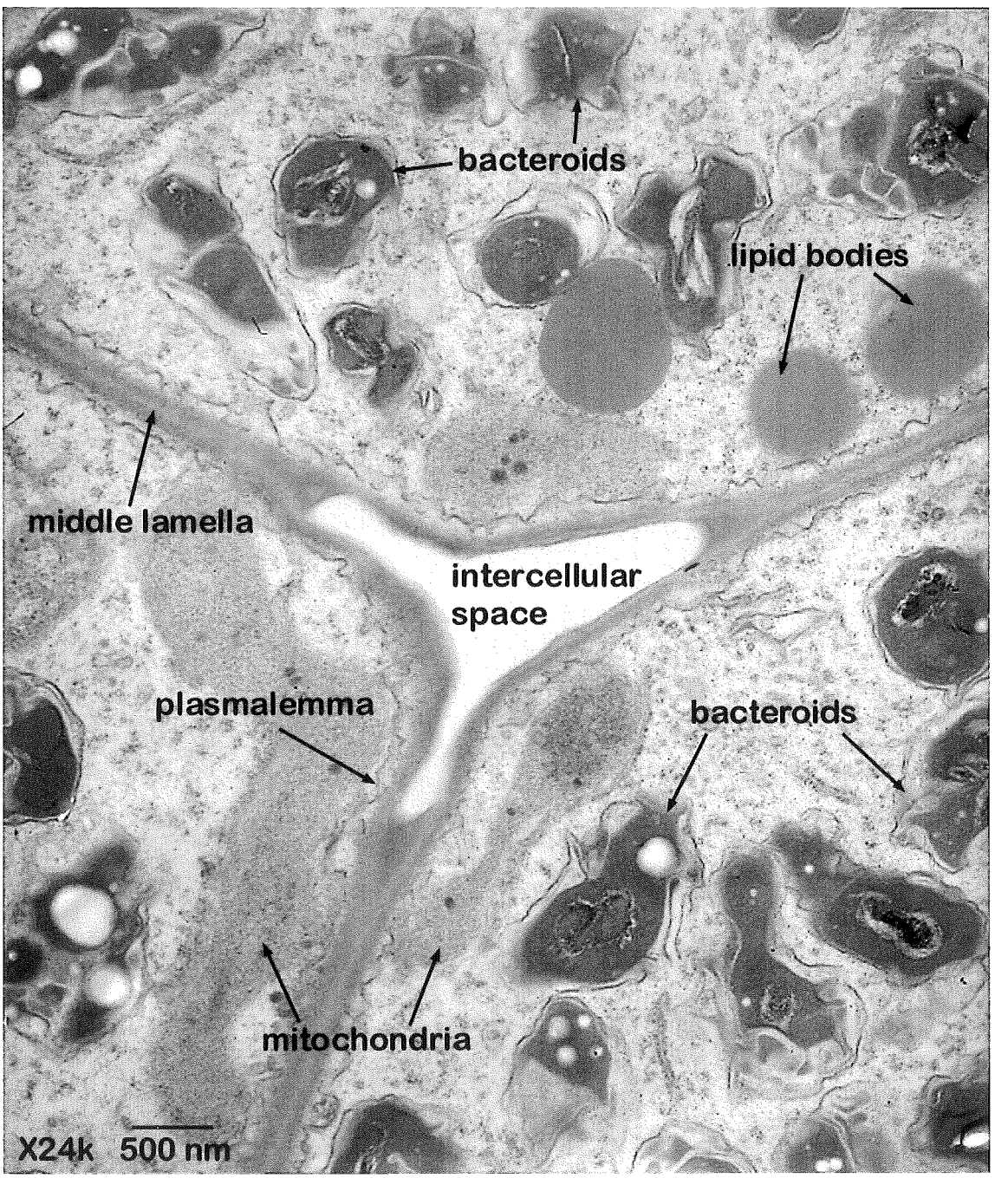
PLATE 20 Transmission electron micrograph at 40,000 X magnification of ultra-thin section of *Pinus radiata* shoot treated with 500 μM Pb plus 0.5 mM EDTA, for 7 days.



Pinus radiata shoot 500 µM Pb 0.5 mM EDTA

PLATE 20

PLATE 21 Transmission electron micrograph of ultra-thin section of non-Pb treated *Chamaecytisus palmensis* root nodule at 24,000 X magnification.



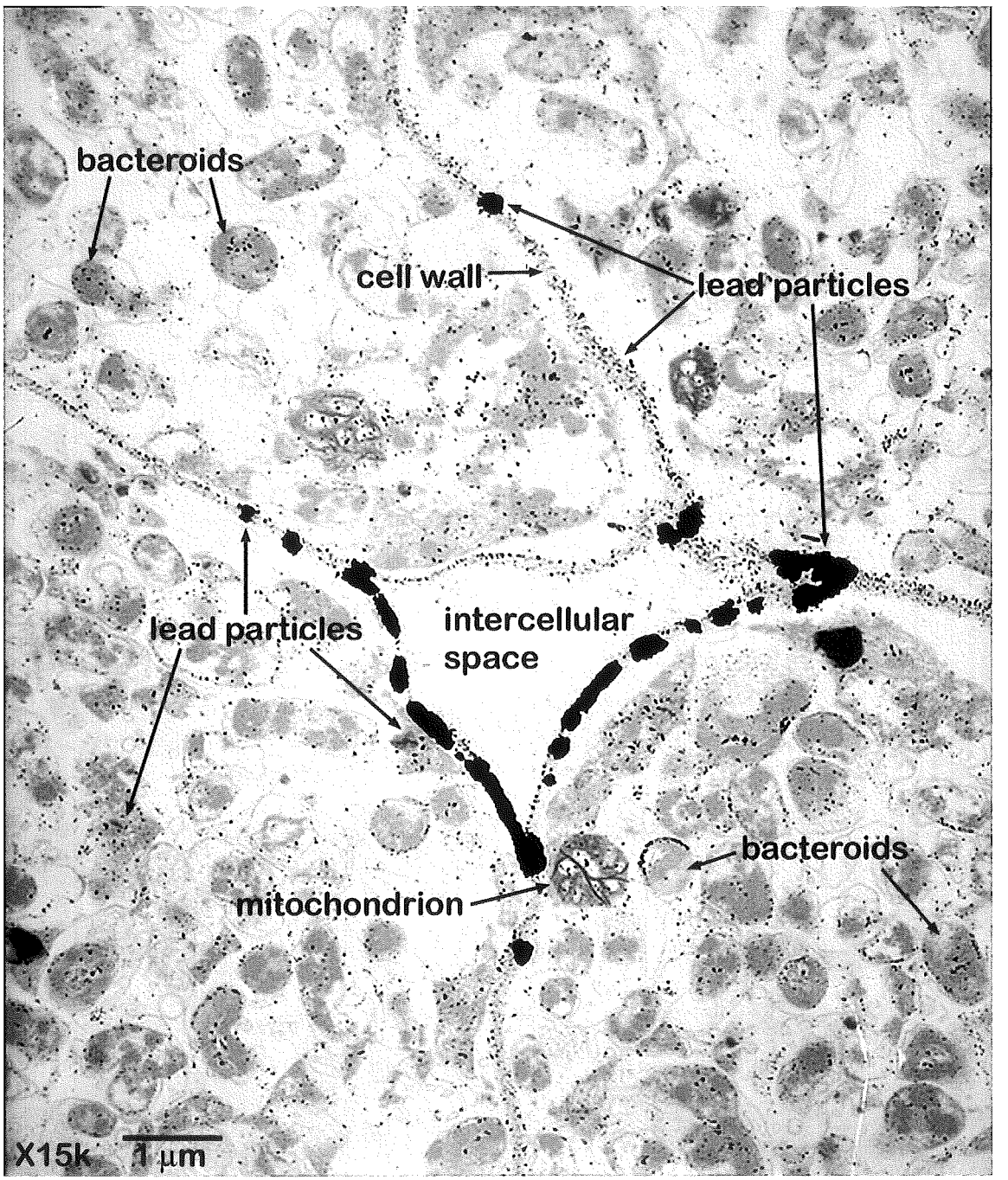
Chamaecytisus palmensis root nodule 0 Pb

PLATE 21

PLATES 22a & 22b Transmission electron micrographs of ultra-thin section of *Chamaecytisus palmensis* root nodule treated with 500 μM Pb, for 7 days.

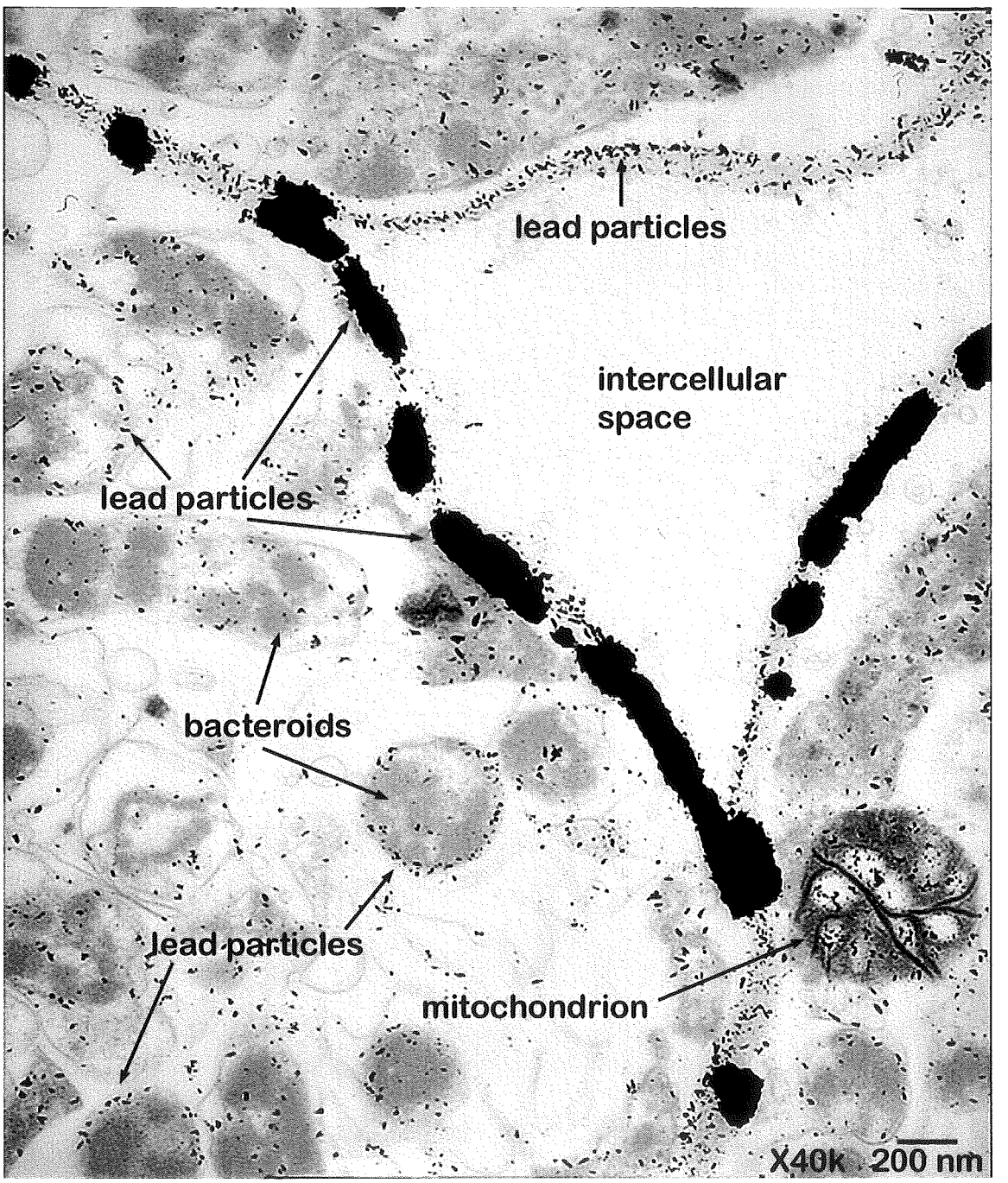
(22a) 15,000 X magnification

(22b) 40,000 X magnification



Chamaecytisus palmensis root nodule 500 µM Pb

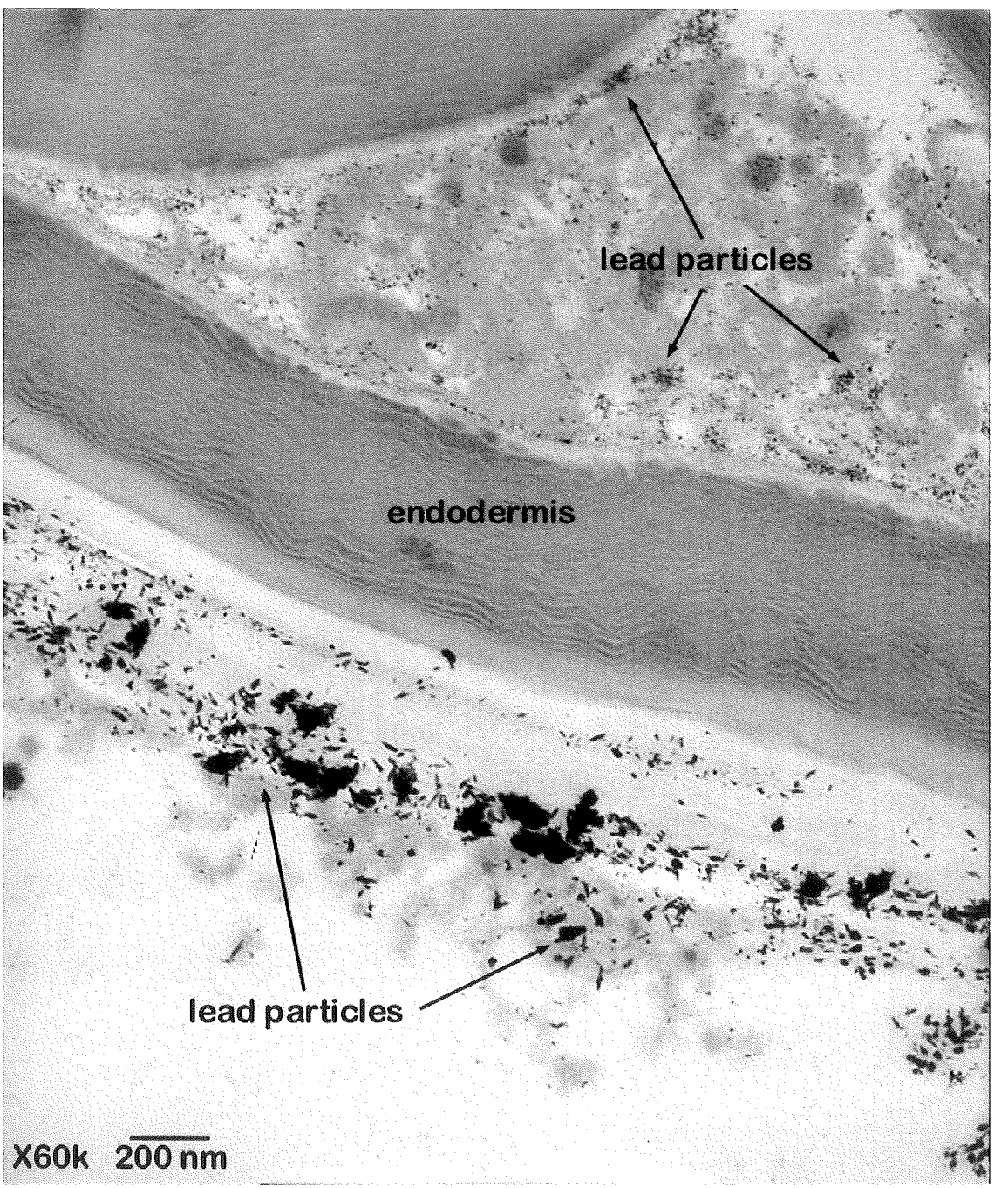
PLATE 22a



Chamaecytisus palmensis root nodule 500 µM Pb

PLATE 22b

PLATE 23 Transmission electron micrograph at 60,000 X magnification of ultra-thin section of *Chamaecytisus palmensis* root nodule treated with 500 μM Pb, for 7 days.



Chamaecytisus palmensis root nodule 500 µM Pb

PLATE 23

Pb particles are in evidence on both sides of the endodermis but very small, finely dispersed Pb grains appear on one side only, as the endodermis appears to have effectively excluded some particles on the basis of size. No Pb particles appear anywhere within the endodermis tissue.

Section of C. palmensis root nodule tissue exposed to 500 μM Pb plus 0.5 mM H-EDTA (Plate 24).

At 30,000X magnification, there is considerably less detail in this section compared with previous root nodule images and this may have been caused by one, or more, of several factors. The root nodule may have been in natural decline at the point of preparation for electron microscopy, the plant may have been in decline at the conclusion of the experiment due to the high level of chelation, or the H-EDTA may have specifically affected the root nodule detrimentally. Despite this, a small number of very fine Pb grains appear to have become associated with the cell wall (Plate 24).

Section of C. palmensis root nodule tissue exposed to 500 μM Pb plus 0.5 mM EDTA (Plate 25).

At 12,000X magnification, this image resembles plate 21, the non-Pb exposed root nodule tissue. The cellular constituents were intact and no evidence could be found of Pb deposition, despite examination at higher magnification (Plate 25).

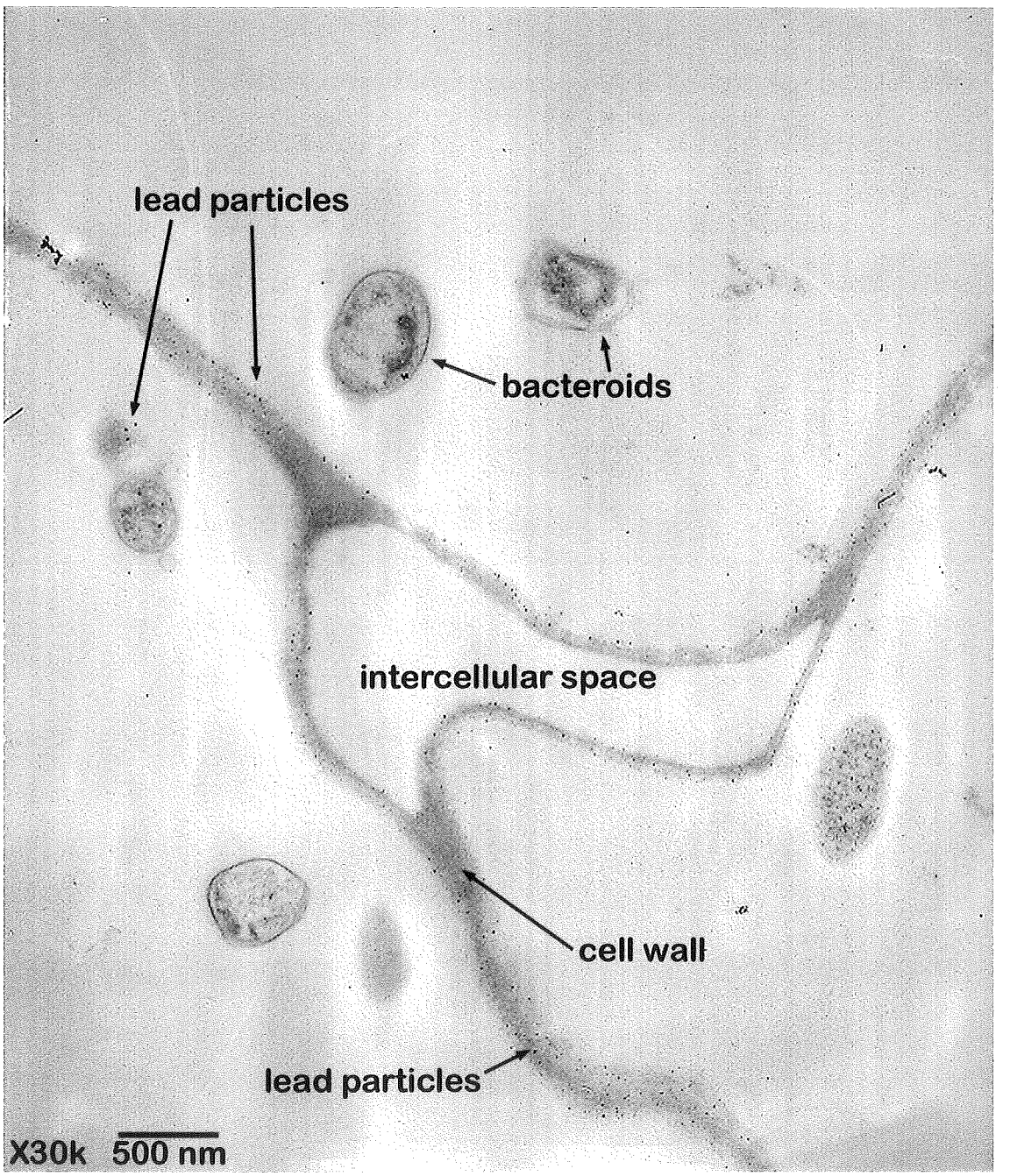
Section of non Pb-exposed C. palmensis root tissue (Plate 26).

At 10,000X magnification, these cells were highly vacuolated and therefore probably quite mature. A more or less continuous layer of tannin is evident in two of the cells (Plate 26).

Sections of C. palmensis root tissue exposed to 500 μM Pb (Plates 27-29).

Here the images depict cells that lacked cellular constituents, appeared highly vacuolated and were therefore probably quite mature. Three cells can be seen surrounding an intercellular space, at 50,000X magnification (Plate 27).

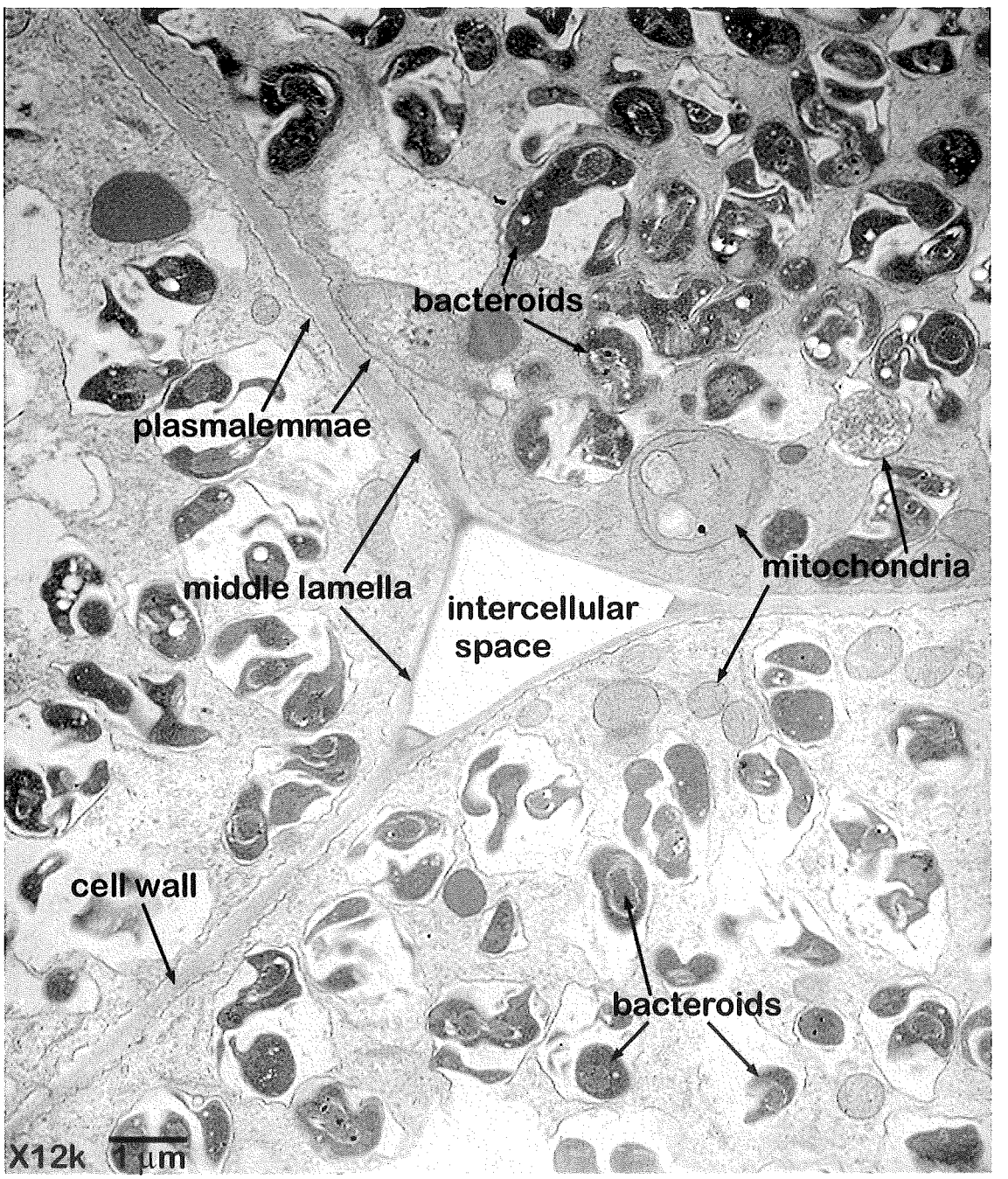
PLATE 24 Transmission electron micrograph at 30,000 X magnification of ultra-thin section of *Chamaecytisus palmensis* root nodule treated with 500 μM Pb plus 0.5 mM H-EDTA, for 7 days.



Chamaecytisus palmensis root nodule 500 µM Pb + 0.5 mM H-EDTA

PLATE 24

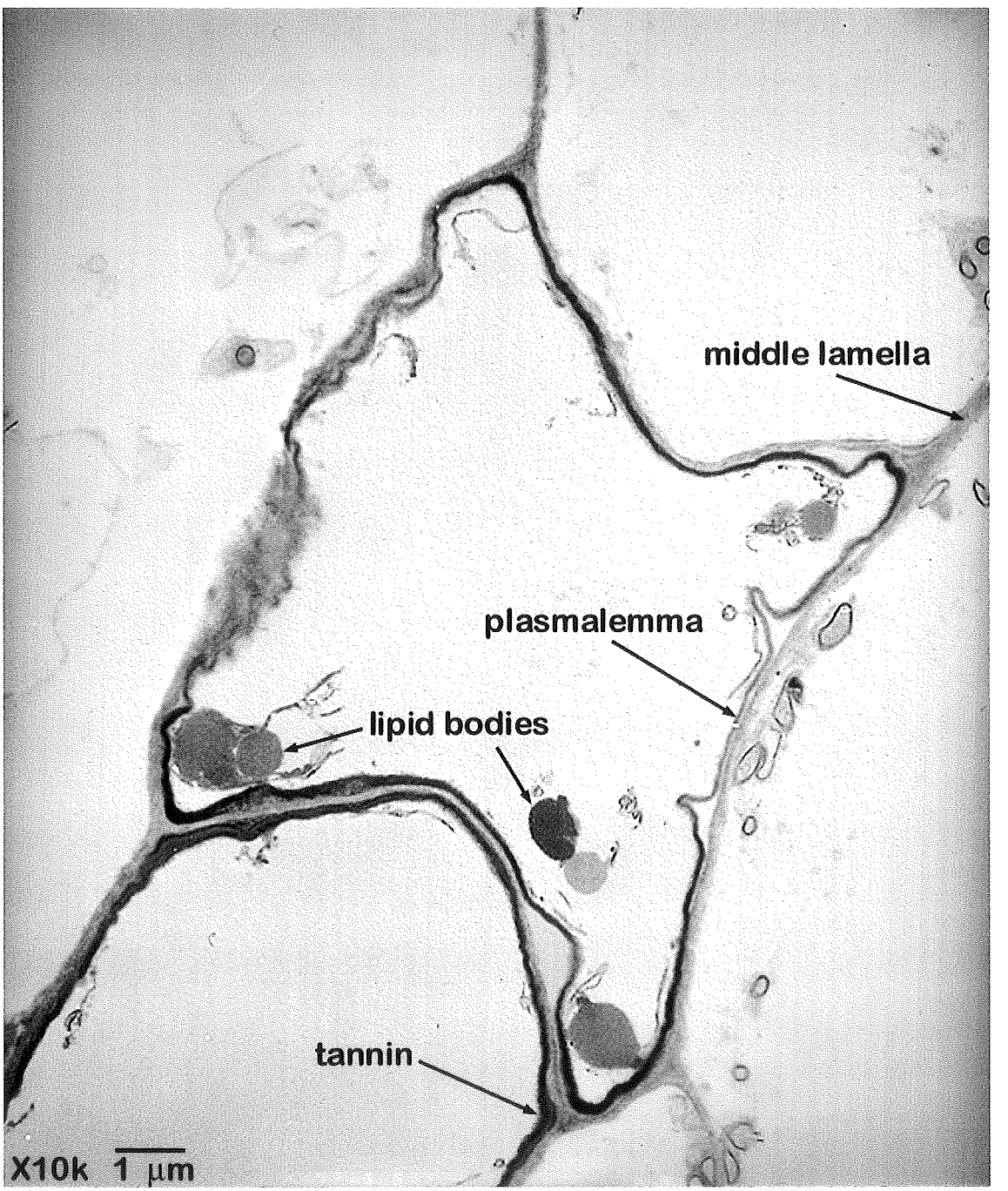
PLATE 25 Transmission electron micrograph at 12,000 X magnification of ultra-thin section of *Chamaecytisus palmensis* root nodule treated with 500 μM Pb plus 0.5 mM EDTA, for 7 days.



Chamaecytisus palmensis root nodule 500 µM Pb 0.5 mM EDTA

PLATE 25

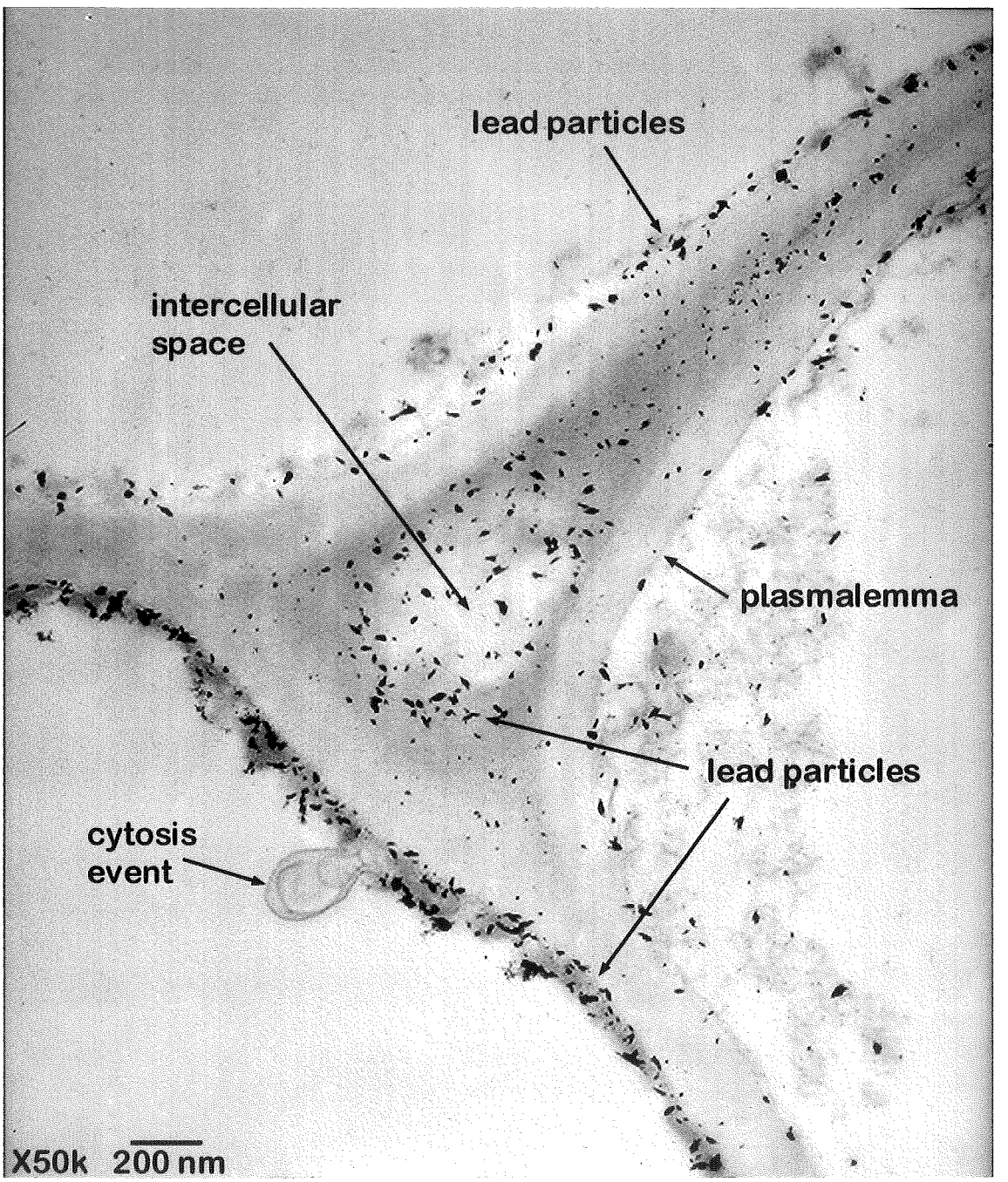
PLATE 26 Transmission electron micrograph at 10,000 X magnification of ultra-thin section of non-Pb treated *Chamaecytisus palmensis* root.



Chamaecytisus palmensis root 0 Pb

PLATE 26

PLATE 27 Transmission electron micrograph at 50,000 X magnification of ultra-thin section of *Chamaecytisus palmensis* root treated with 500 μM Pb, for 7 days.



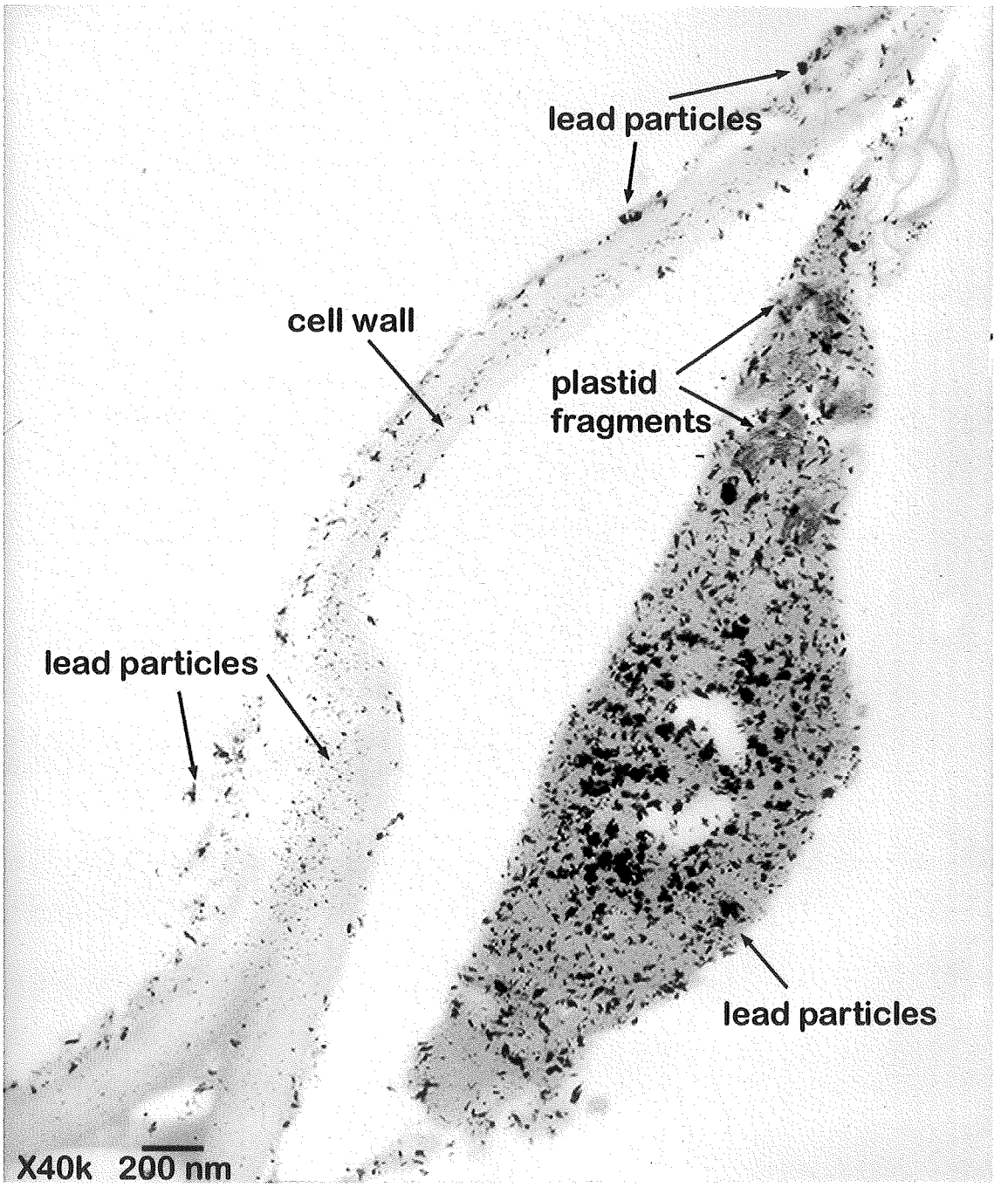
Chamaecytisus palmensis root 500 µM Pb

PLATE 27

PLATES 28a & 28b Transmission electron micrographs of ultra-thin section of *Chamaecytisus palmensis* root treated with 500 μM Pb for 7 days.

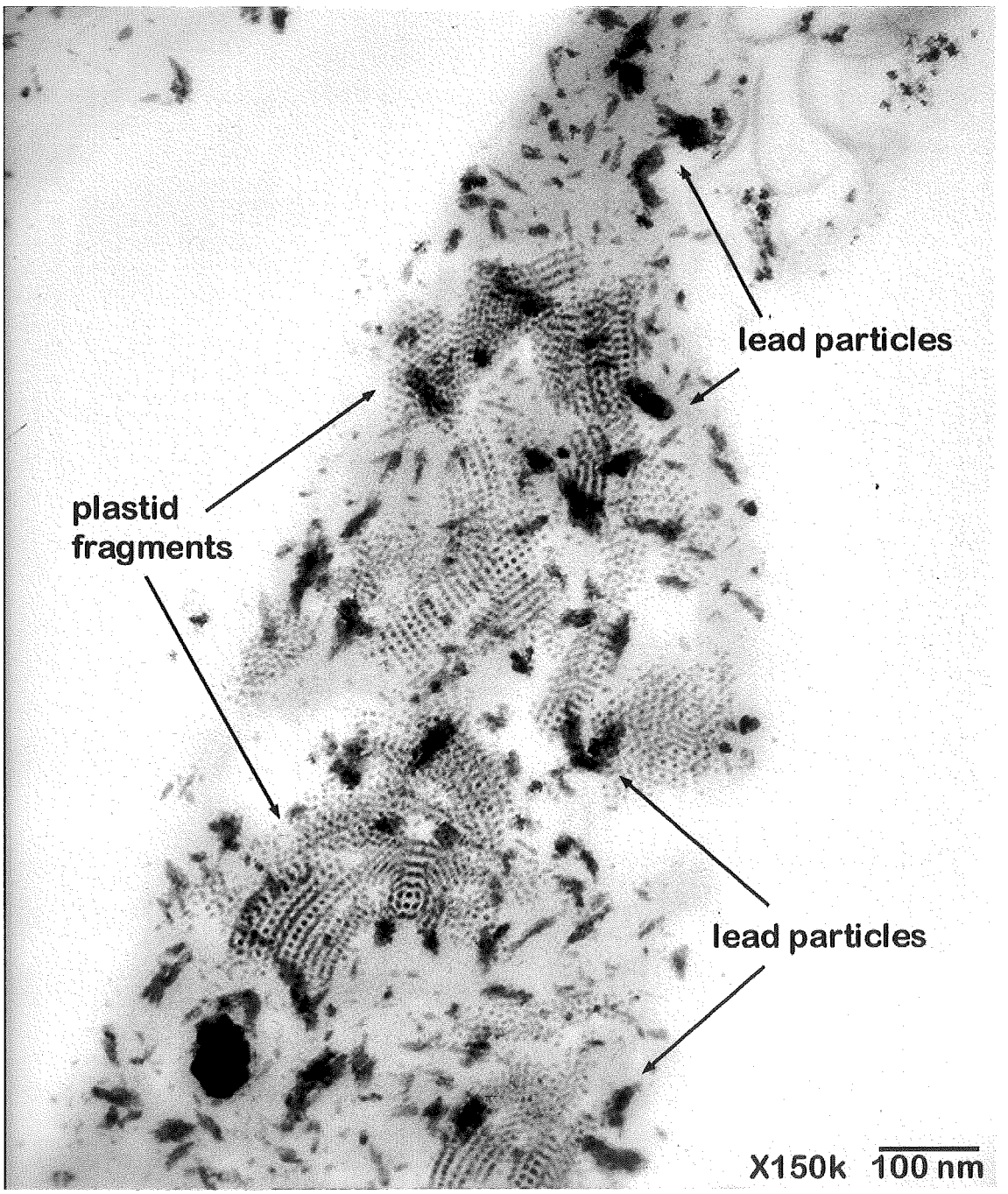
(28a) 40,000 X magnification

(28b) 150,000 X magnification



Chamaecytisus palmensis root 500 µM Pb

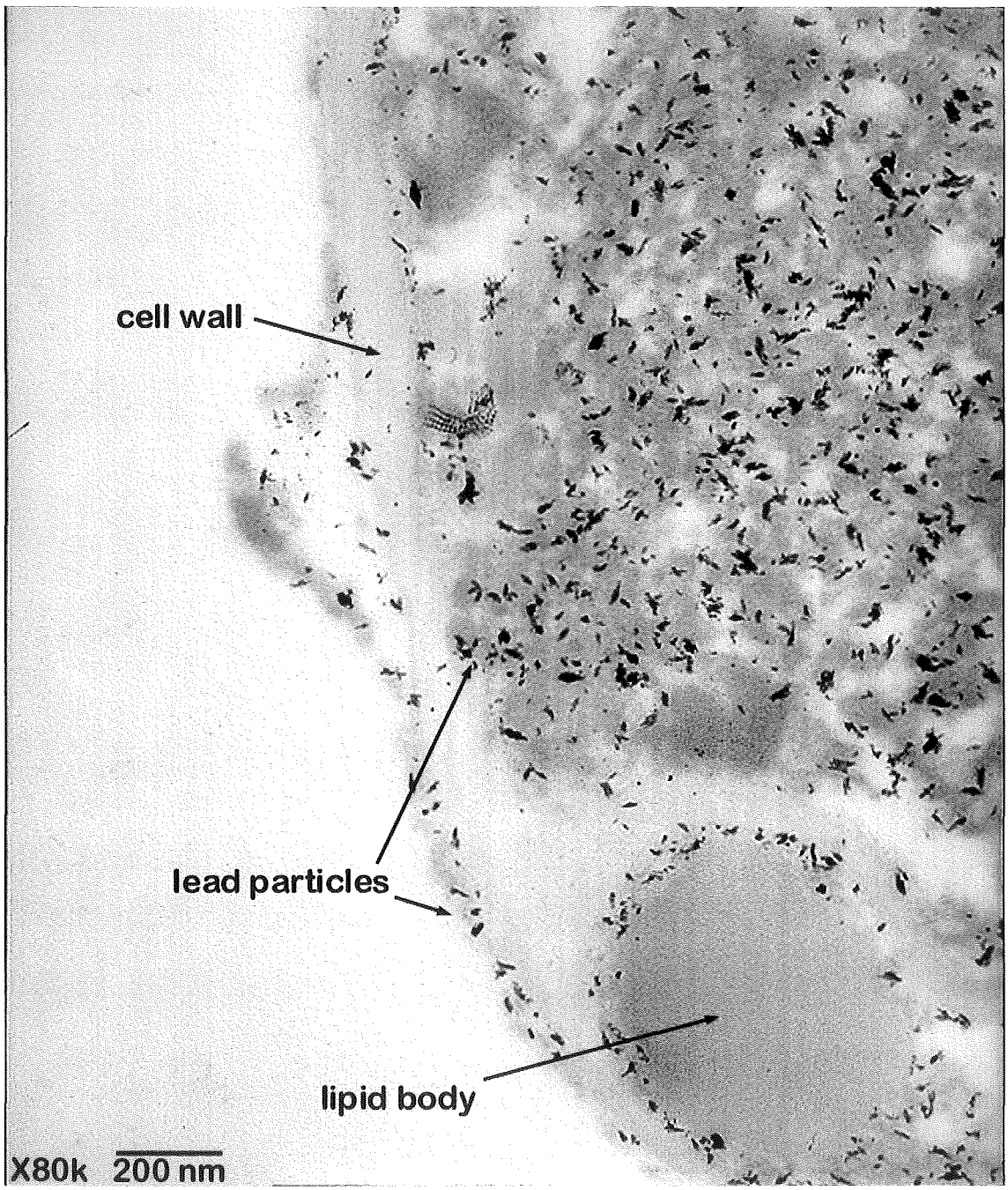
PLATE 28a



Chamaecytisus palmensis root 500 µM Pb

PLATE 28b

PLATE 29 Transmission electron micrograph at 80,000 X magnification of ultra-thin section of *Chamaecytisus palmensis* root treated with 500 μM Pb, for 7 days.



Chamaecytisus palmensis root 500 µM Pb

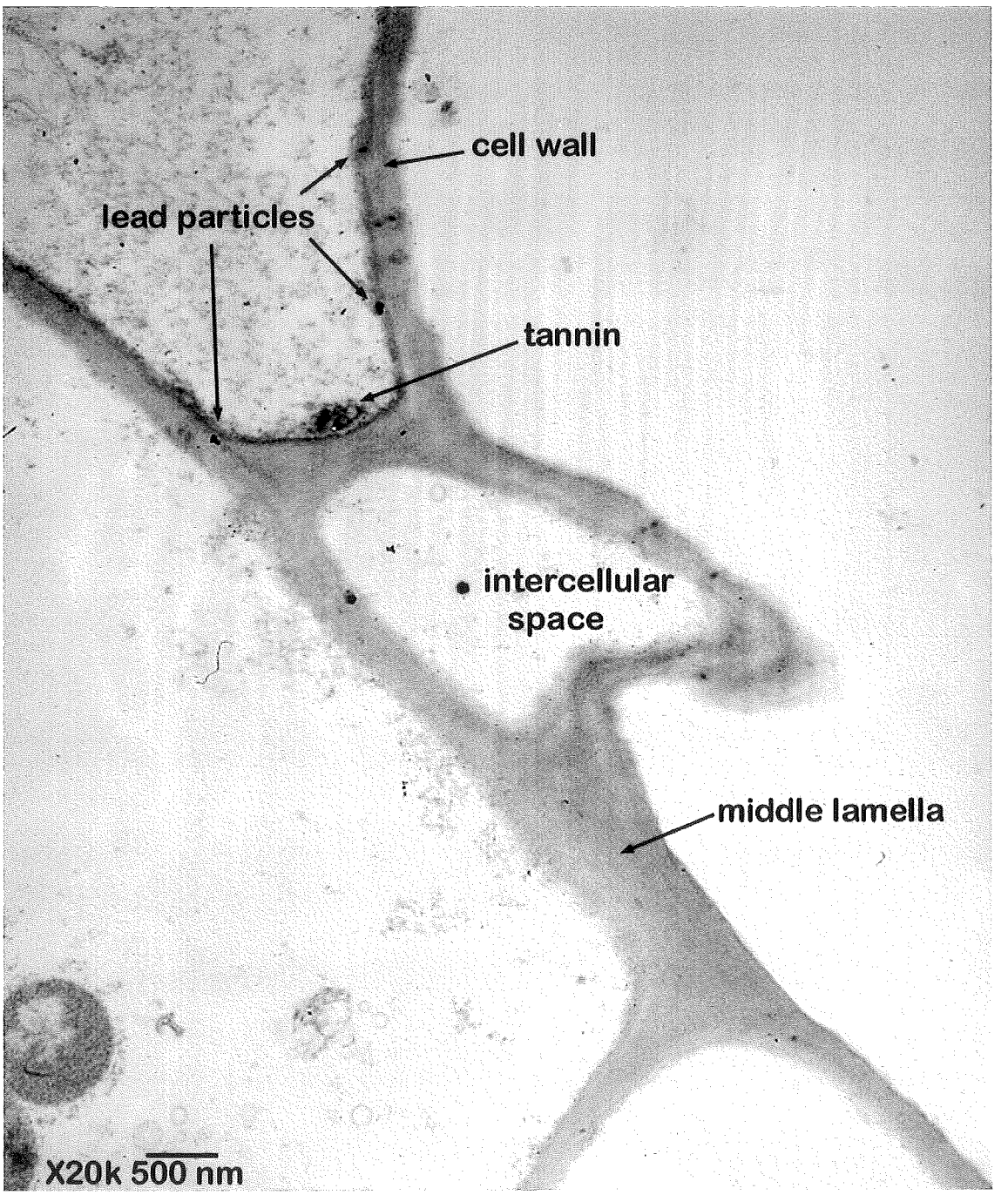
PLATE 29

Heavy deposits of relatively large Pb grains were found in the intercellular space and regions of the middle lamella. In addition, significant amounts of Pb were found in the material adhering to the cell wall but little Pb was deposited within the matrix of the wall itself. Interestingly, this section revealed the occurrence of a cytosis event, adjacent to quite a heavy deposition of Pb. A section of cell wall and portion of cellular debris can be seen at 40,000X magnification (Plate 28a). Pb deposition has occurred continuously along the cell wall with larger particles attached to the outer layers of the wall and finer particles embedded in the middle lamella. The piece of cellular debris, which may be some kind of tannin-like deposit and which might have originally been attached to the cell wall, contains very heavy deposits of large Pb particles. Material similar to this was seen in plate 27, where it also contained heavy deposits of Pb. At 150,000X magnification, the crystalline nature of the plastid fragments, which are only just visible in plate 28a, can clearly be seen, as well as the heterogeneous nature of the deposited Pb particles (Plate 28b). The disintegration of the plastid was probably linked to the maturity of the cell. More of the intracellular material with a high affinity for Pb is seen at 80,000X magnification, where the sharp, jagged nature of the densely deposited Pb particles can be discerned (Plate 29). In the cell wall, the Pb particles are associated with only the outer layers while the lipid body at lower right has also effectively excluded them.

Section of C. palmensis root tissue exposed to 500 μM Pb plus 0.5 mM H-EDTA (Plate 30).

At 20,000X magnification, this image has similarities to plate 24, (root nodule tissue exposed to H-EDTA). There is a lack of detail in the section which may indicate that the tissue was in decline when it was prepared, or that the relatively high concentration of H-EDTA may have detrimentally affected the cells. However, a modest number of putative Pb particles were found in regions of the cell wall (Plate 30).

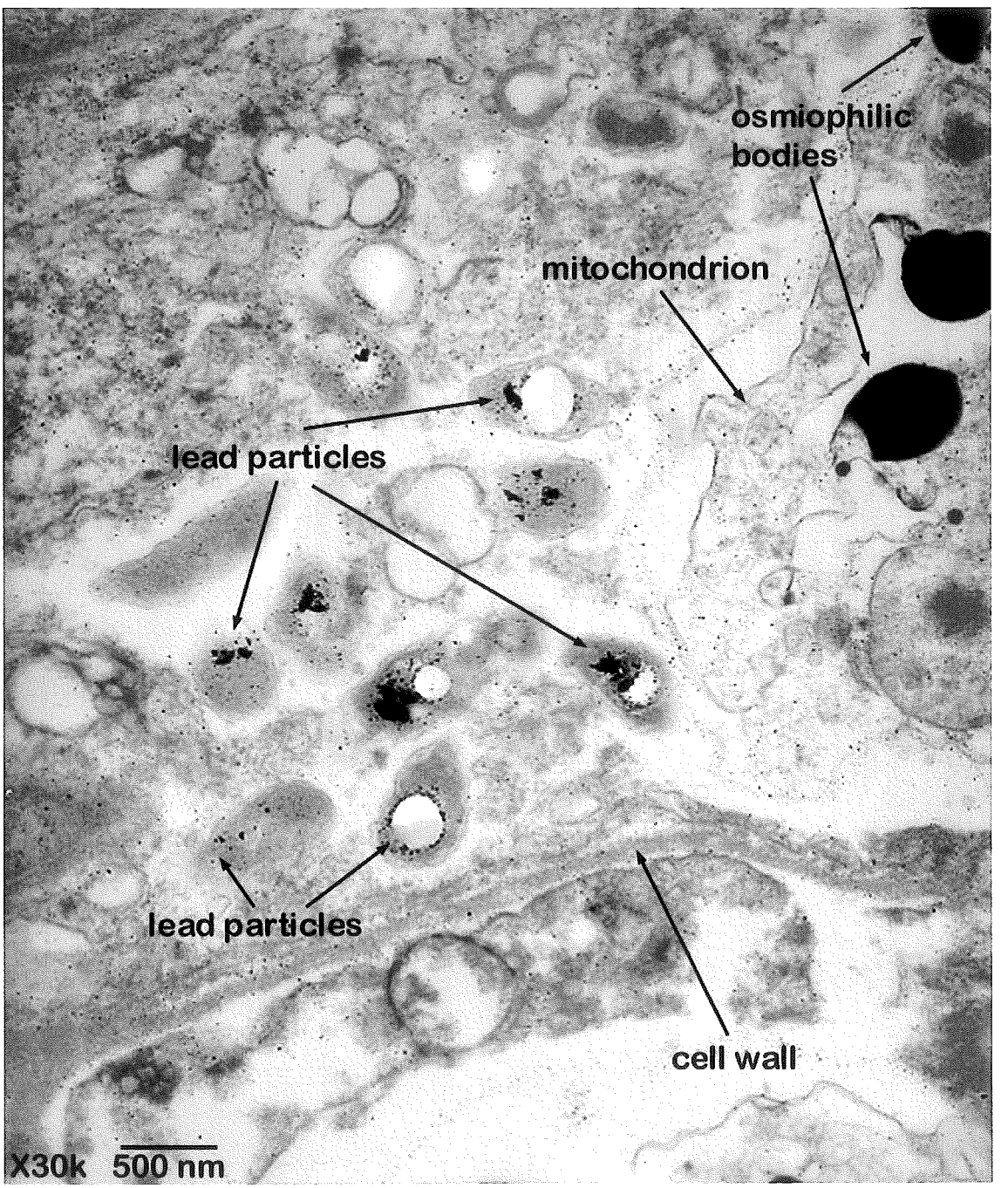
PLATE 30 Transmission electron micrograph at 20,000 X magnification of ultra-thin section of *Chamaecytisus palmensis* root treated with 500 μM Pb plus 0.5 mM H-EDTA, for 7 days.



Chamaecytisus palmensis root 500 µM Pb + 0.5 mM H-EDTA

PLATE 30

PLATE 31 Transmission electron micrograph at 30,000 X magnification of ultra-thin section of *Chamaecytisus palmensis* root treated with 500 μM Pb plus 0.5 mM EDTA, for 7 days.



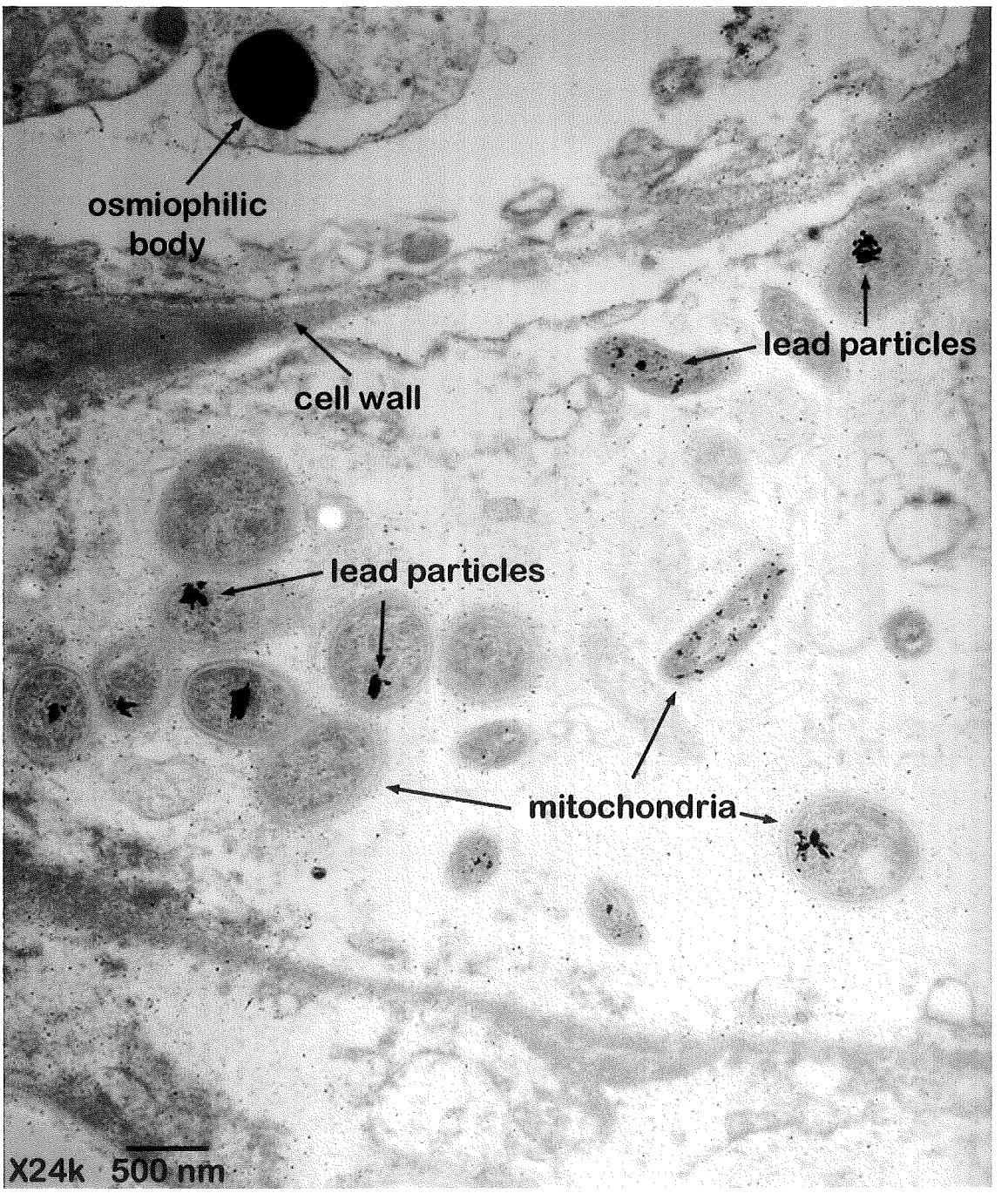
Chamaecytisus palmensis root 500 µM Pb + 0.5 mM EDTA

PLATE 31

PLATES 32a & 32b Transmission electron micrographs of ultra-thin section of *Chamaecytisus palmensis* root treated with 500 μM Pb plus 0.5 mM EDTA, for 7 days.

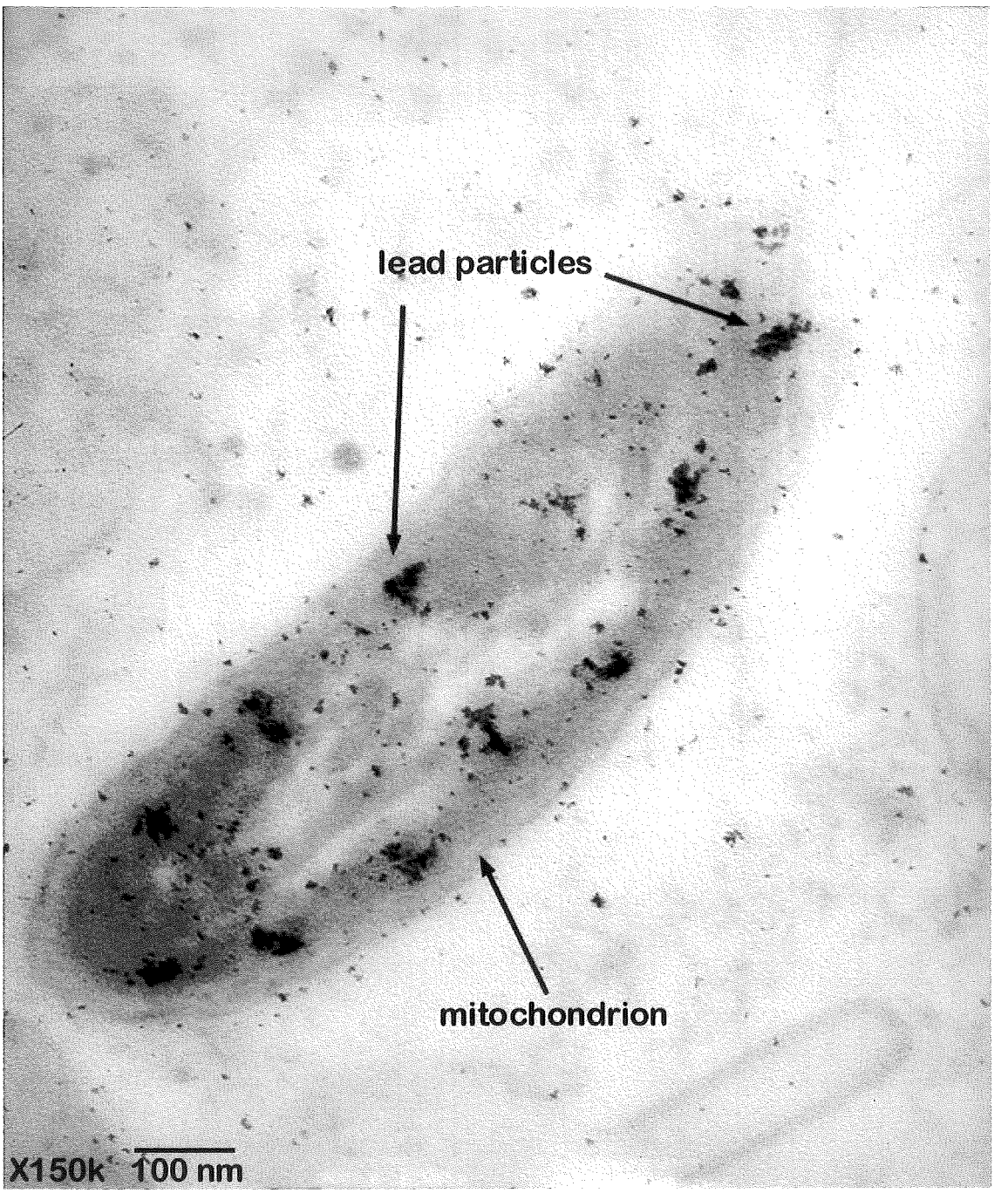
(32a) 24,000 X magnification

(32b) 150,000 X magnification



Chamaecytisus palmensis root 500 µM Pb + 0.5 mM EDTA

PLATE 32a



Chamaecytisus palmensis root 500 µM Pb + 0.5 mM EDTA

PLATE 32b

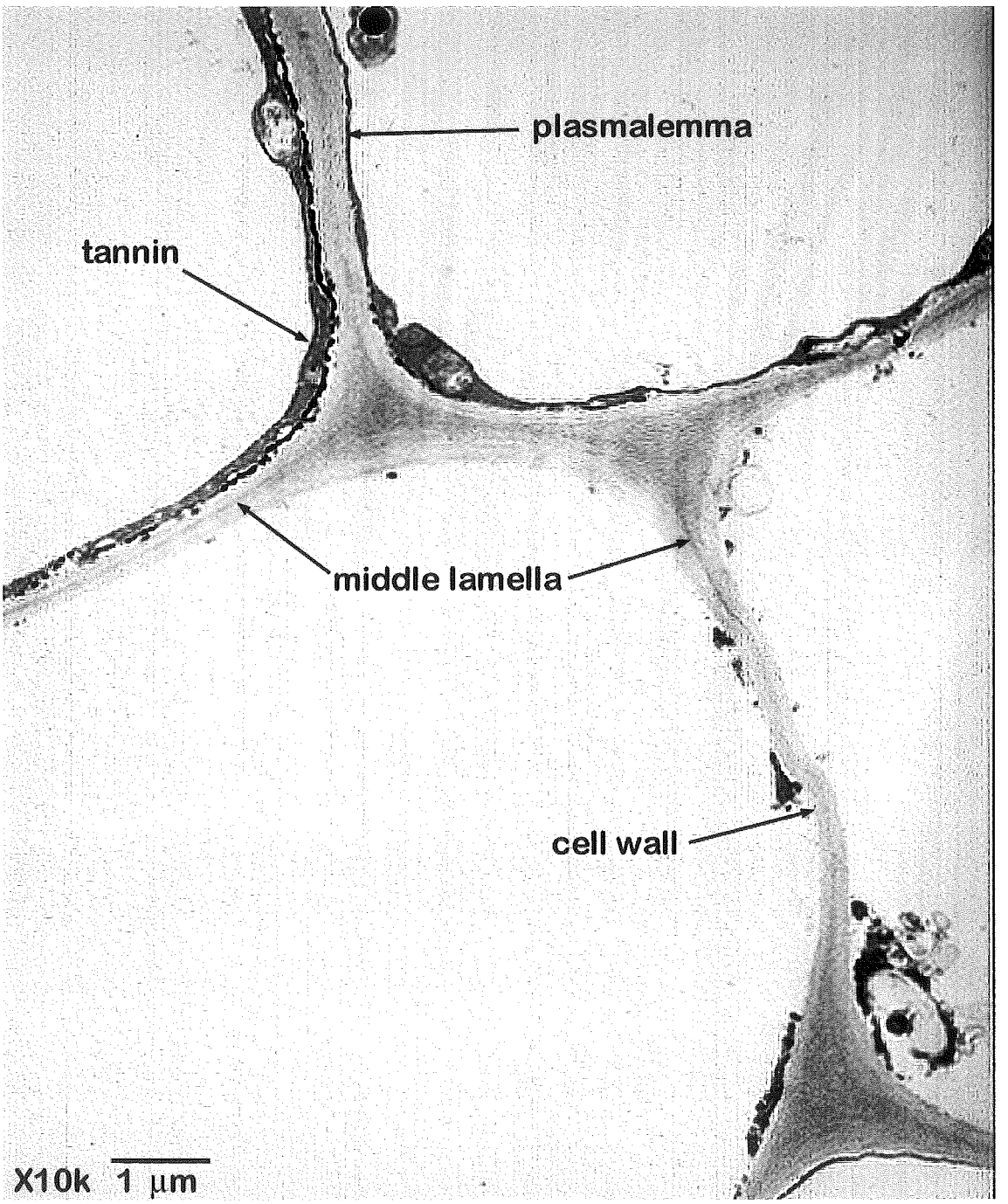
Sections of C. palmensis root tissue exposed to 500 μM Pb plus 0.5 mM EDTA (Plates 31 & 32).

At the junction of two cells, at 30,000X magnification, small Pb particles are dispersed randomly at low density throughout the uppermost cell, while higher density, localised Pb deposits are seen associated with the structures in the centre of the image (Plate 31). These structures are possibly amyloplasts in which case the round clear bodies within them are starch grains. The electron dense bodies seen at upper right, labeled as osmiophilic, are cellular components that have an affinity for osmium tetroxide, which contributes to their very dark appearance. Practically no Pb was found in association with the cell wall. At 24,000X magnification, the principal feature is a cell containing numerous mitochondria (Plate 32a). Pb deposition has occurred almost entirely within the tissues of the mitochondria and very little can be detected elsewhere. Where the Pb has been taken up in the mitochondria, it has done so both as discrete clumps of large particles, and as evenly dispersed fine grains. At 150,000X magnification, one mitochondrion is seen in detail, with evenly dispersed Pb grains embedded in it (Plate 32b). In this image it can be seen that not all the Pb is contained within the mitochondrion, small amounts are in evidence in the region surrounding it. Despite the presence of Pb within the mitochondria, morphologically these organelles appeared to be intact.

Sections of non-Pb exposed C. palmensis shoot tissue (Plates 33 & 34).

Tannin deposits can be seen at the periphery of several highly vacuolated cells, lacking cellular components, at 10,000X magnification (Plate 33). Within a cell containing a chloroplast at 60,000X magnification, the structure of the thylakoid membranes can be clearly seen, interspersed with electron-dense plastoglobuli, or lipid droplets (Plate 34). In this image, very fine, evenly dispersed dark grains can be detected within the tissues of the chloroplast. In this section these are clearly not Pb particles, but it is essential to note their presence for comparison with Pb-treated tissues.

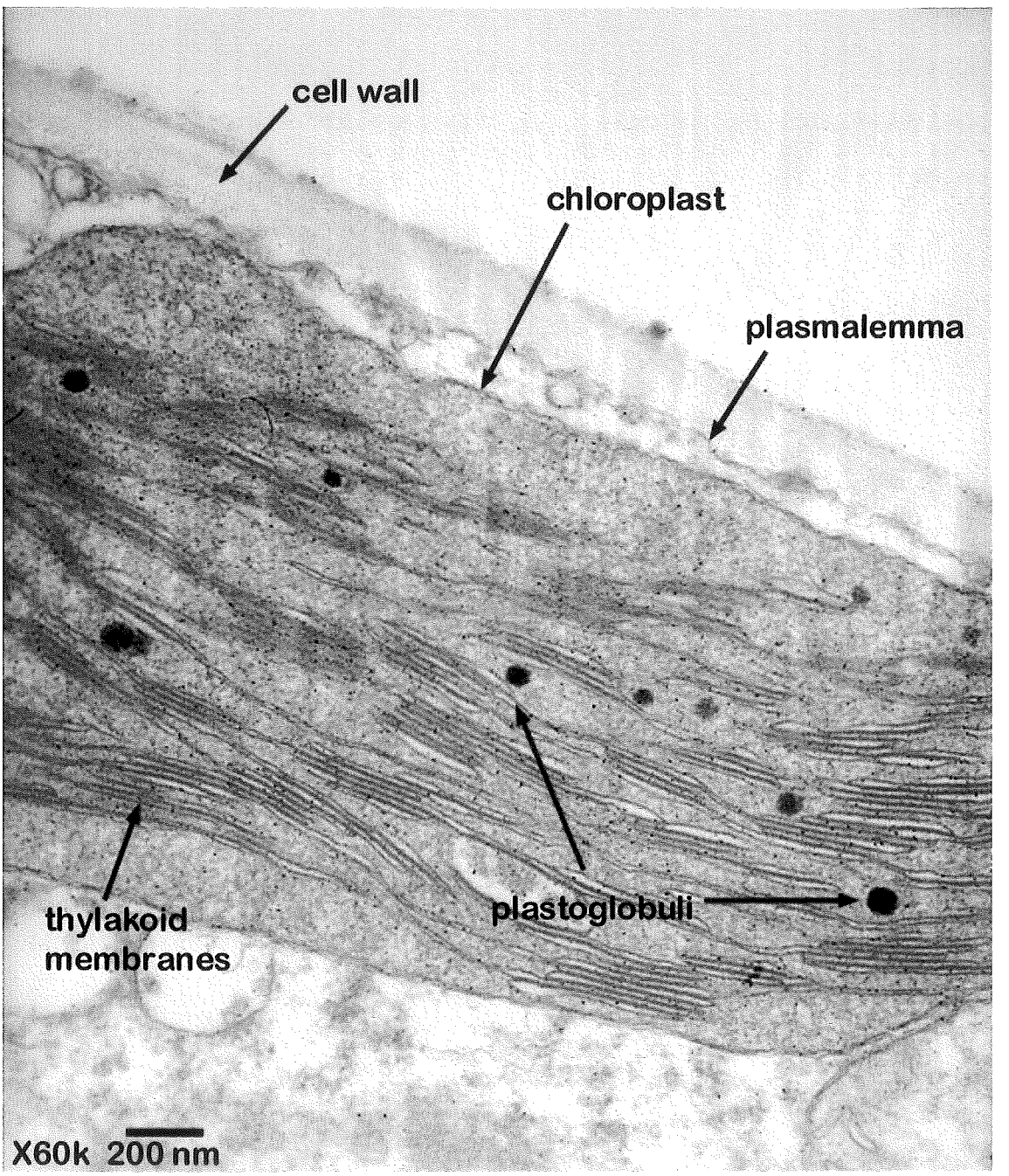
PLATE 33 Transmission electron micrograph at 10,000 X magnification of ultra-thin section of non Pb-treated *Chamaecytisus palmensis* shoot.



Chamaecytisus palmensis shoot 0 Pb

PLATE 33

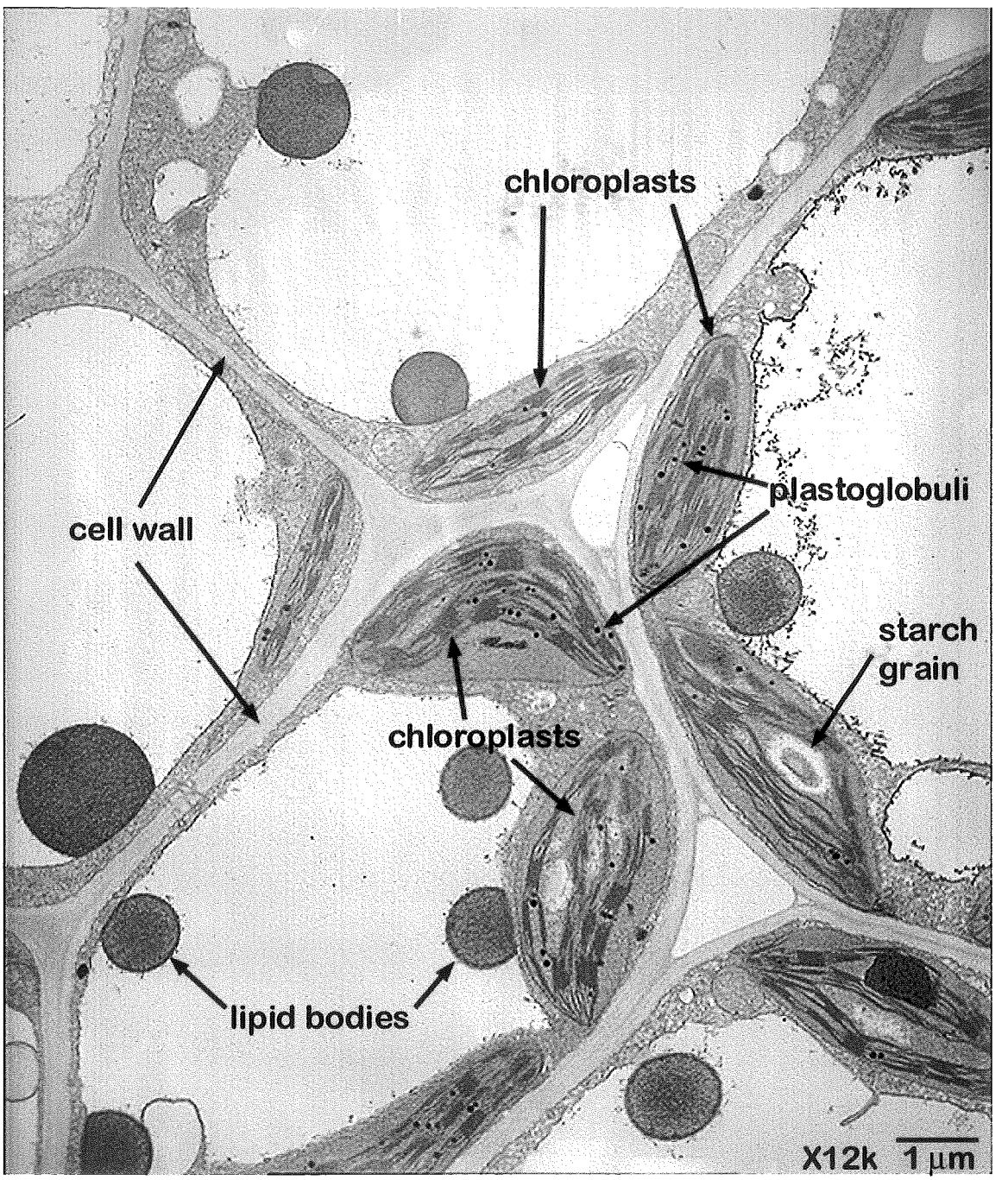
PLATE 34 Transmission electron micrograph of ultra-thin section of non Pb-treated *Chamaecytisus palmensis* shoot at 60,000 X magnification.



Chamaecytisus palmensis shoot 0 Pb

PLATE 34

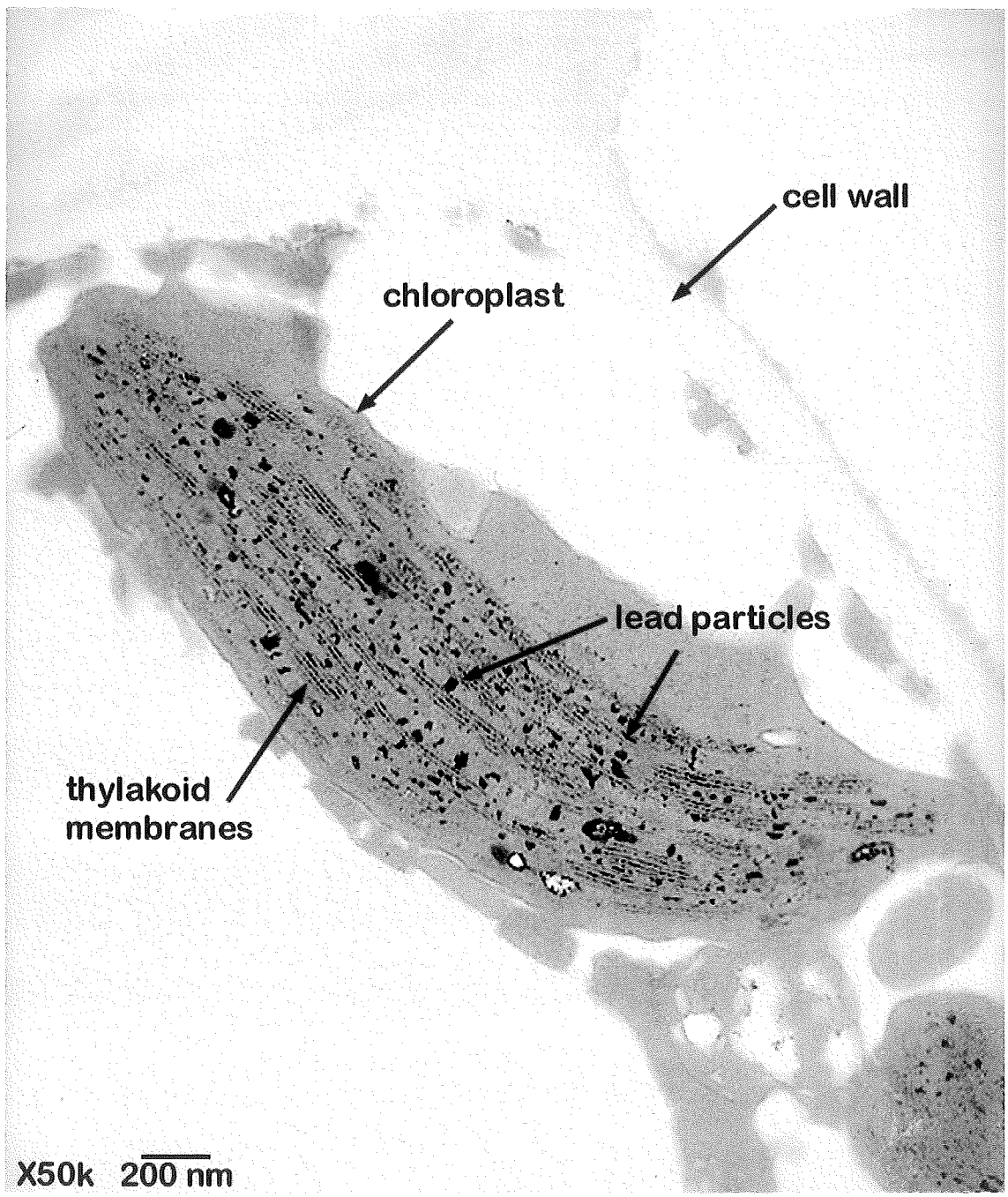
PLATE 35 Transmission electron micrograph at 12,000 X magnification of ultra-thin section of *Chamaecytisus palmensis* shoot treated with 500 μM Pb, for 7 days.



Chamaecytisus palmensis shoot 500 µM Pb

PLATE 35

PLATE 36 Transmission electron micrograph at 50,000 X magnification of ultra-thin section of *Chamaecytisus palmensis* shoot treated with 500 μM Pb, for 7 days.



Chamaecytisus palmensis shoot 500 µM Pb

PLATE 36

Sections of C. palmensis shoot tissue exposed to 500 μM Pb (Plates 35 & 36). Multiple chloroplasts can be seen within several moderately vacuolated cells, at 12,000X magnification (Plate 35). Tannin deposits are evident at the periphery of one of the cells and within the chloroplasts plastoglobuli are present. Pb particles were not detected in these organelles at higher magnification. However, at 50,000X magnification, is a chloroplast that appears to have taken up Pb particles (Plate 36). Interspersed with the thylakoid membranes, Pb particles of various sizes can clearly be seen. The morphology of this chloroplast seems somewhat altered in comparison with the chloroplasts in plate 35. Here the thylakoid membranes are darker in patches and plastoglobuli are not readily discernible. Possibly this is due to the incorporation of the Pb particles but it may be due to the maturity of the cell. It is interesting to note the relatively large size of the Pb particles in plate 36, as this type of unchelated Pb was rarely seen in shoot material elsewhere.

Sections of C. palmensis shoot tissue exposed to 500 μM Pb plus 0.5 mM H-EDTA (Plate 37).

At 15,000X magnification, primary pit fields connect three cells which were highly vacuolated, quite mature, and probably components of the vascular system, perhaps xylem elements (Plate 37a). Very fine Pb particles have become embedded at high density in the primary cell wall regions which comprise the pit complexes. Very little Pb seems to have become associated with the unmodified regions of the cell wall elsewhere in this section. At 50,000X magnification, the very fine nature of the Pb grains deposited within the primary pit field can be seen (Plate 37b).

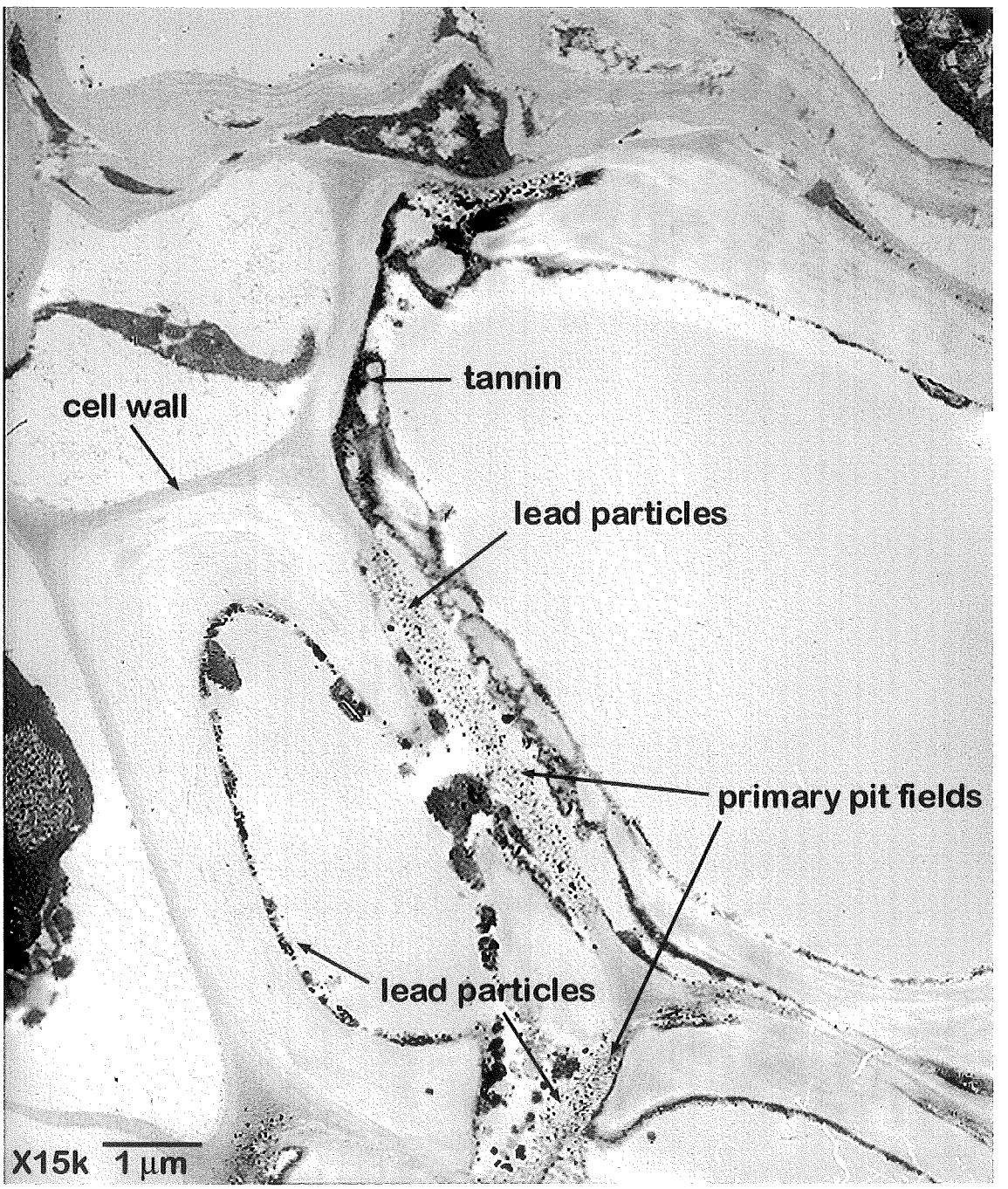
Sections of C. palmensis shoot tissue exposed to 500 μM Pb plus 0.5 mM EDTA (Plates 38 & 39).

At 120,000X magnification, the junction of two chloroplast-containing cells can be seen (Plate 38). Pb appears to have been deposited as extremely fine particles in the outer layers of the cell walls and also within the chloroplasts.

PLATES 37a & 37b Transmission electron micrographs of ultra-thin section of *Chamaecytisus palmensis* shoot treated with 500 μM Pb plus 0.5 mM H-EDTA, for 7 days.

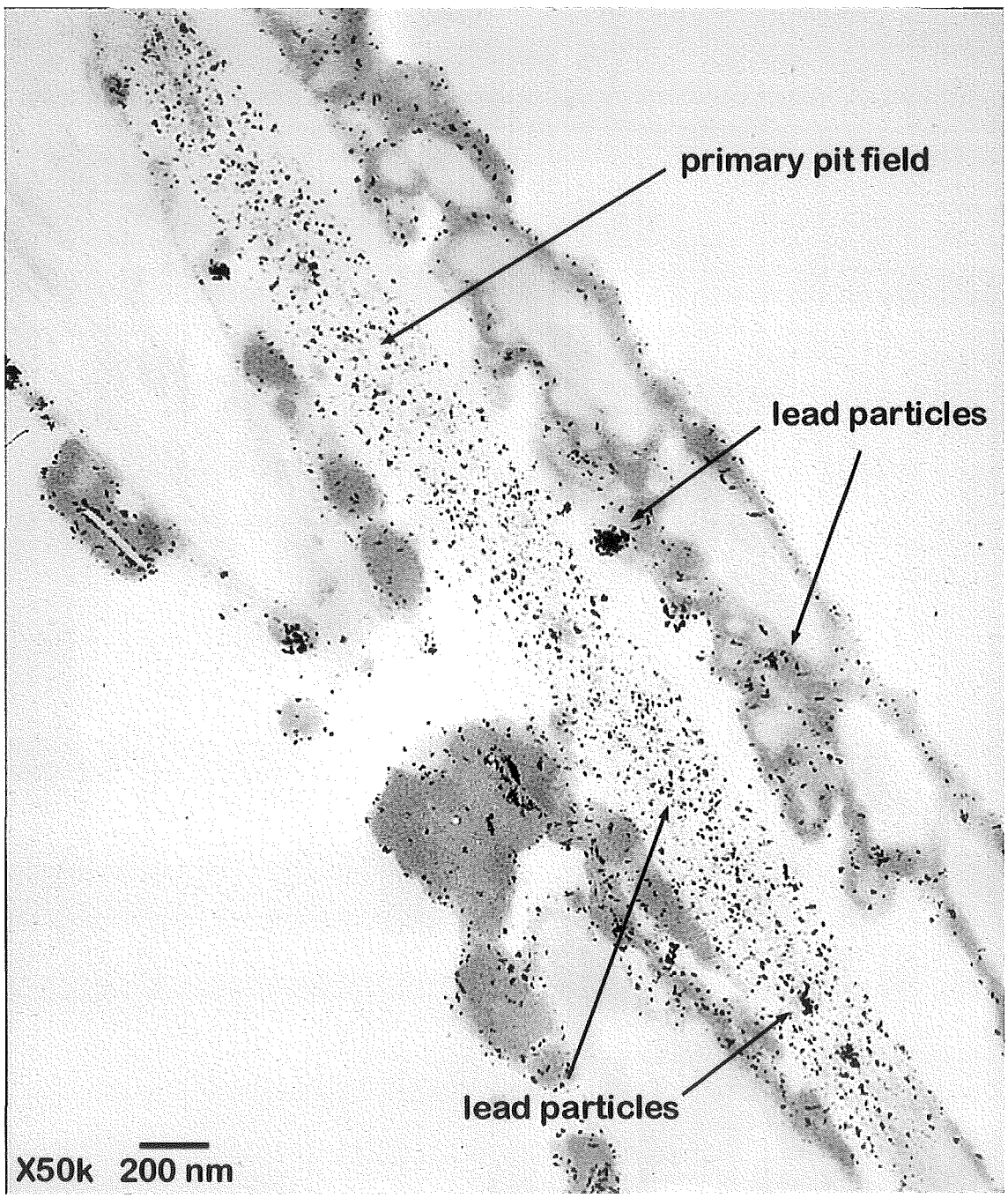
(37a) 15,000 X magnification

(37b) 50,000 X magnification



Chamaecytisus palmensis shoot 500 µM Pb 0.5 mM H-EDTA

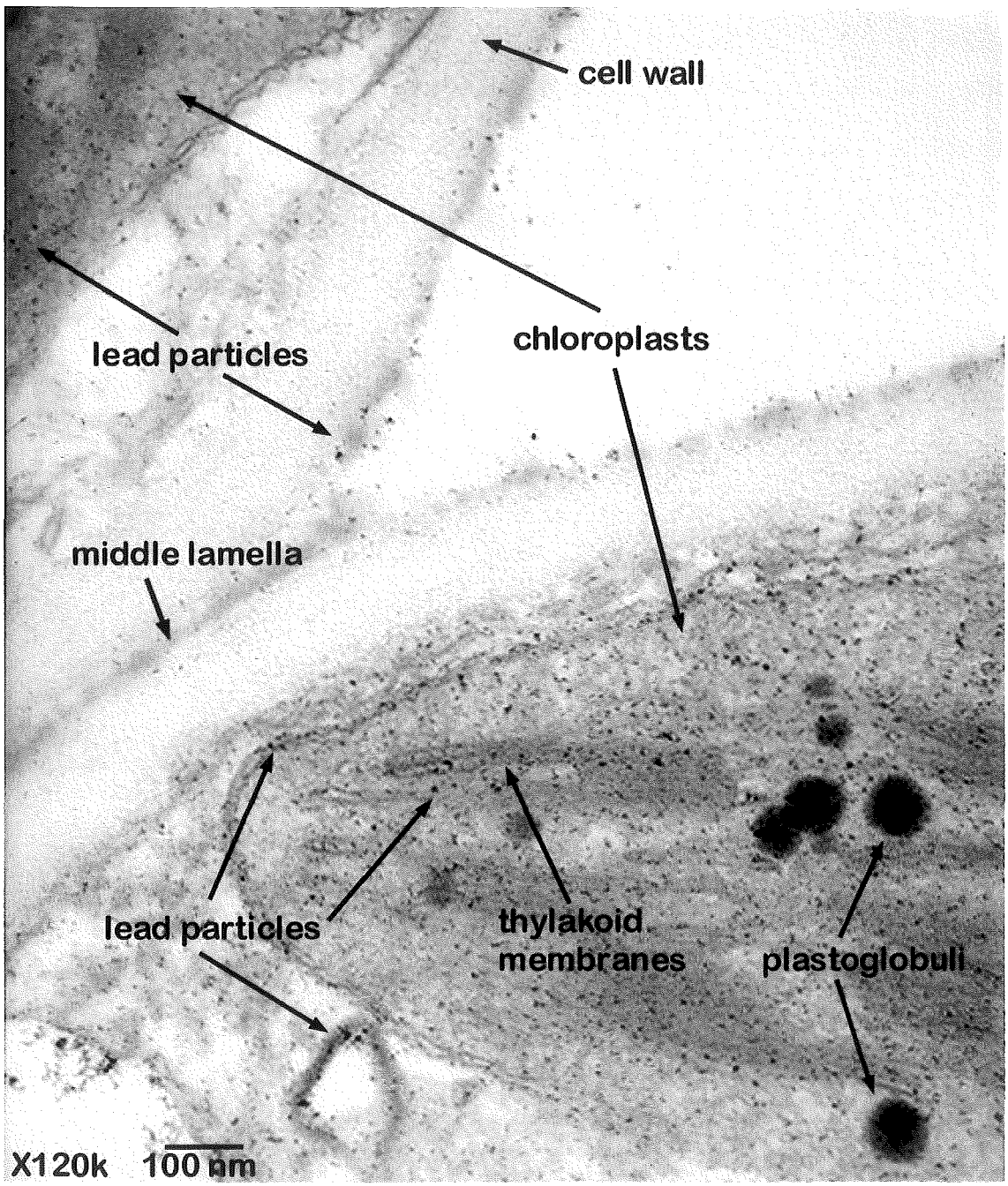
PLATE 37a



Chamaecytisus palmensis shoot 500 µM Pb 0.5 mM H-EDTA

PLATE 37b

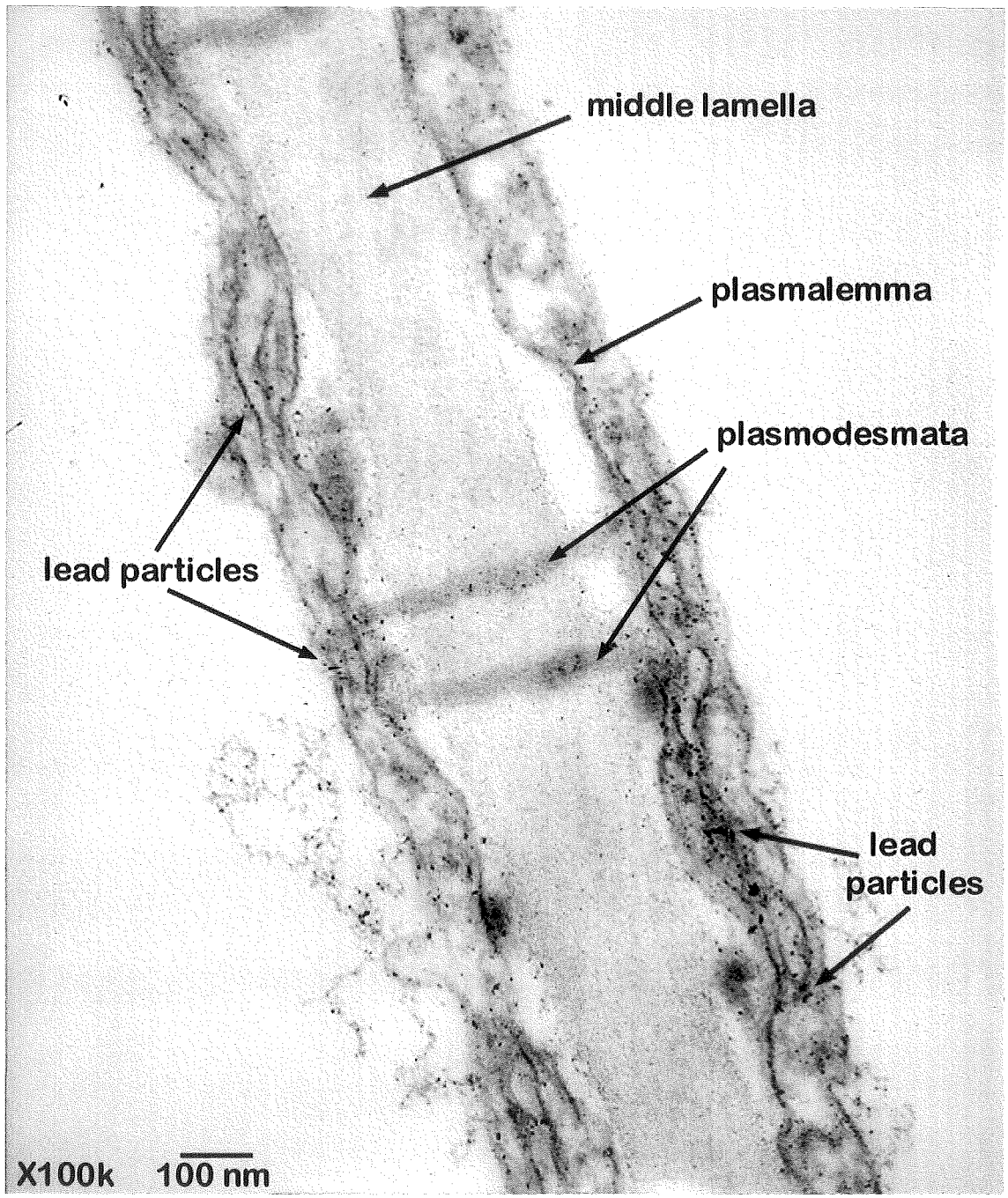
PLATE 38 Transmission electron micrograph at 120,000 X magnification of ultra-thin section of *Chamaecytisus palmensis* shoot treated with 500 μM Pb plus 0.5 mM EDTA, for 7 days.



Chamaecytisus palmensis shoot 500 µM Pb 0.5 mM EDTA

PLATE 38

PLATE 39 Transmission electron micrograph at 100,000 X magnification of ultra-thin section of *Chamaecytisus palmensis* shoot treated with 500 μM Pb plus 0.5 mM EDTA, for 7 days.



Chamaecytisus palmensis shoot 500 µM Pb 0.5 mM EDTA

PLATE 39

Although similarities exist between this image and plate 34 (non-Pb exposed shoot), careful consideration and careful examination at low and high magnification have led to the conclusion that this is the manner in which Pb chelated with EDTA is deposited within some *C. palmensis* shoot tissues. At 100,000X magnification (Plate 39), fine Pb grains are evident in the outer layers of a cell wall containing plasmodesmata, the intracellular material lining the periphery of the cell, and also embedded within the plasmodesmata. No large Pb particles were observed.

(6) ACID PHOSPHATASE

6.1 Qualitative assay

After 5 hours exposure to XP, non Pb-treated seedlings and germinated seeds of *P. radiata* displayed a prominent blue colour associated with the outer epidermal layers of the root tissues (result not shown), indicating the presence of acid phosphatase enzyme. Similarly, non Pb-treated *C. palmensis* seedlings, after 5 hours exposure to XP, displayed prominent blue colouration of outer and inner layers of root cells (result not shown).

After 18 hours exposure to XP in the presence of 20 μM Pb, both *C. palmensis* and *P. radiata* seedlings displayed prominent blue colouration of the roots (result not shown). This indicated that Pb at this concentration had no effect on acid phosphatase activity associated with the root surface. Stereomicroscopic inspection of the *C. palmensis* roots found blue staining in all the root cells including root hairs, with a particularly dark area of staining visible as a central cylinder running down each root. Free-hand sections of these roots showed that the blue staining was occurring mainly at the outer surface. In the *P. radiata* roots the blue staining was also occurring in a layer of outer epidermal cells, including root hairs.

6.2 Quantitative assay

After 6 days, at zero P, in the absence of Pb, the shoots and roots of *P. radiata* seedlings had comparable, low levels of acid phosphatase (Figure 23).

When the seedlings were exposed to 250 or 500 μM Pb, with and without chelation at 0.5 mM, for 7 days, the enzyme activity levels were similar and low for both root and shoot (Figures 24 & 25).

When *P. radiata* clones V, W, X, and T were exposed to 500 μM Pb, with and without chelation at 0.5 mM for 7 days, shoot enzyme activity levels (Figure 26a) were nearly 5 times as high as root enzyme levels (Figure 26b) in the same

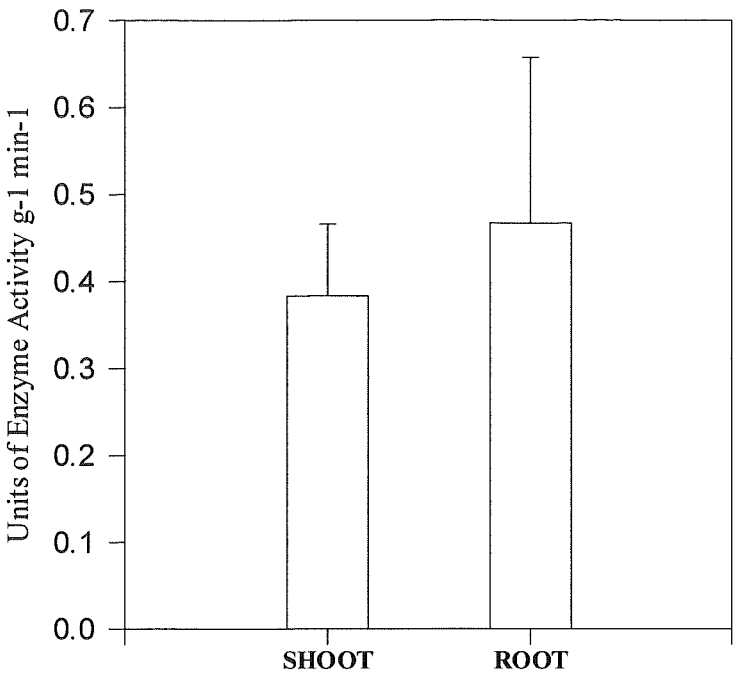


Figure 23. Acid phosphatase activity in extracts from 12 week old seedlings of *Pinus radiata* after 6 days in a zero P environment. Bars represent standard error (n=2).

plants. More variation was apparent within shoot enzyme activity levels than those of root .

After *P. radiata* clones U and S were exposed to 500 μM Pb, with and without chelation at 0.5 mM for 7 days, shoot enzyme activity levels (Figure 27a) were between 4 and 7 times as high as root enzyme activity levels (Figure 27b) in the same plants. Although there was little variation in enzyme activity levels among shoot treatments, shoot enzyme activity levels for clone S were almost twice those of clone U. Little variation occurred in root enzyme activity among treatments or between clones.

Enzyme activity levels of shoots of *C. palmensis* seedlings exposed to zero P, in the absence of Pb, were approximately 16 times as high as root enzyme activity levels in the same plants (Figure 28). The root enzyme activity levels in this tissue were considerably higher than the highest *P. radiata* shoot enzyme activity level recorded.

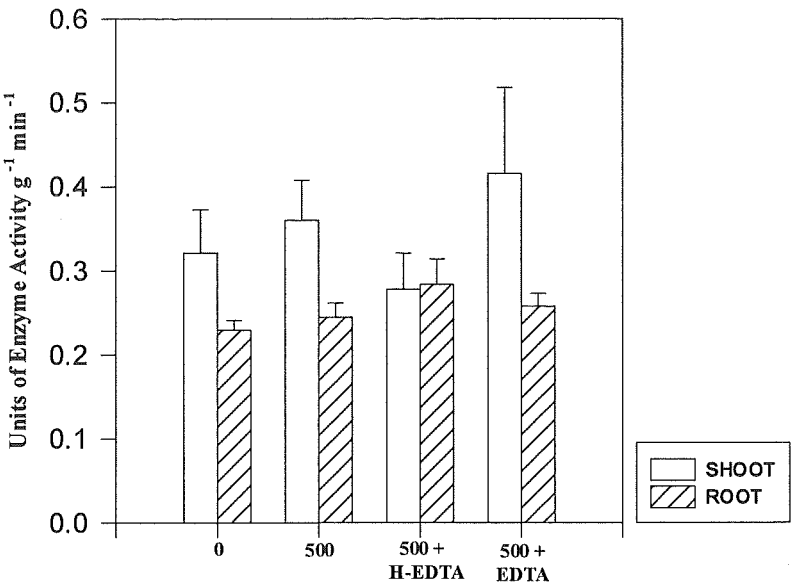


Figure 24. Acid phosphatase activity in extracts from 6 month-old seedlings of *Pinus radiata* after 7 d in $Pb(NO_3)_2$ +/- 0.5 mM H-EDTA or EDTA. Bars represent standard error (n=3).

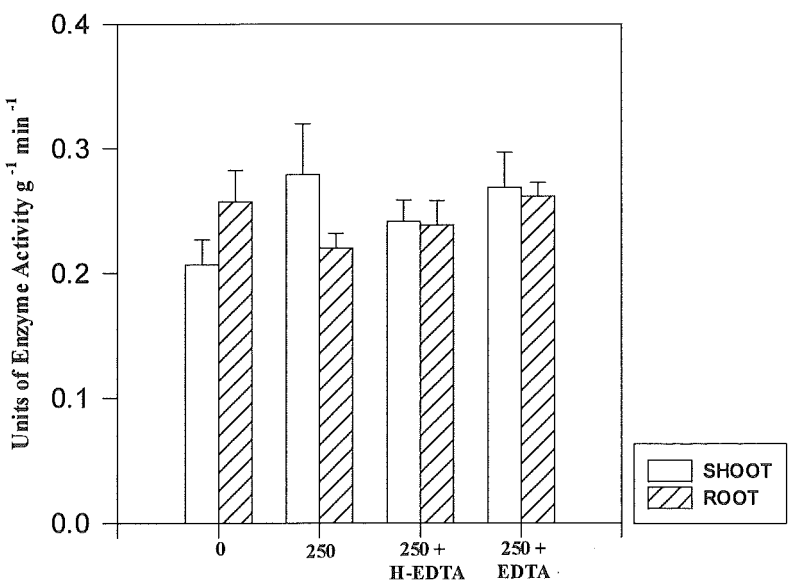


Figure 25. Acid phosphatase activity in extracts from 6 month-old seedlings of *Pinus radiata* after 7 d in $Pb(NO_3)_2$ +/- 0.5 mM H-EDTA or EDTA. Bars represent standard error (n=2 or 3).

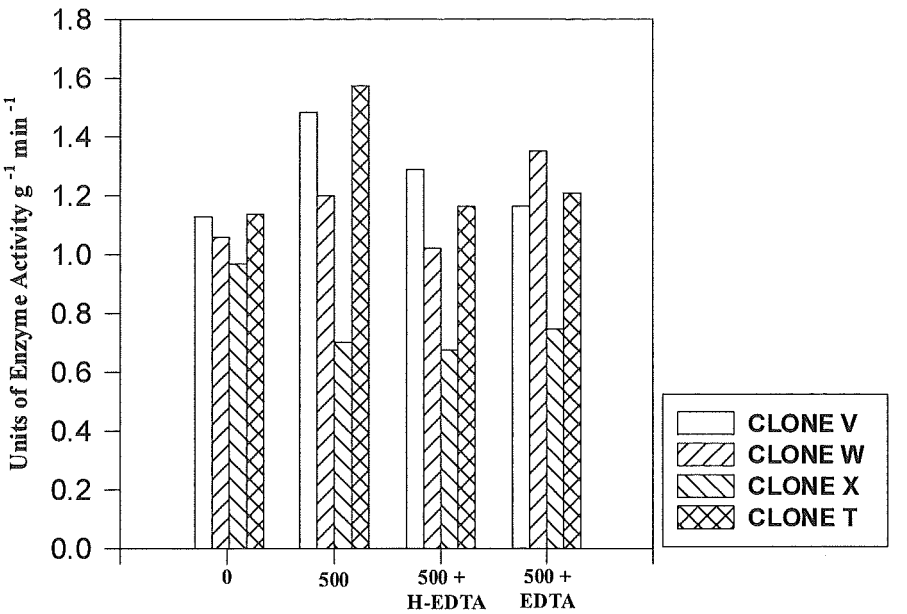


Figure 26a. Shoot acid phosphatase activity in extracts from 6 month-old clones of *Pinus radiata* after 7 d in $Pb(NO_3)_2$ +/- 0.5 mM H-EDTA or EDTA.

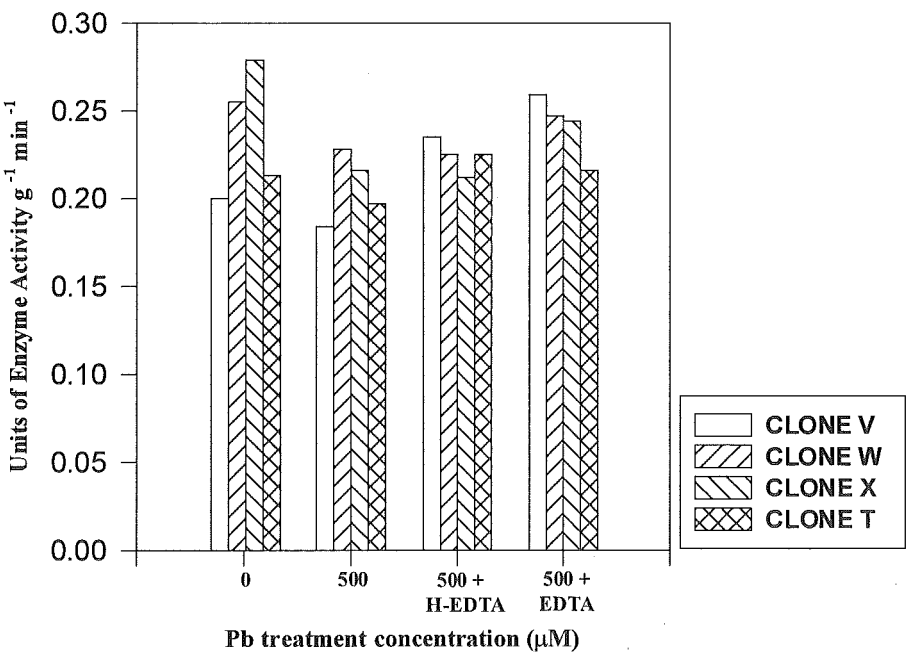


Figure 26b. Root acid phosphatase activity in extracts from 6 month-old clones of *Pinus radiata* after 7 d in $Pb(NO_3)_2$ +/- 0.5 mM H-EDTA or EDTA.

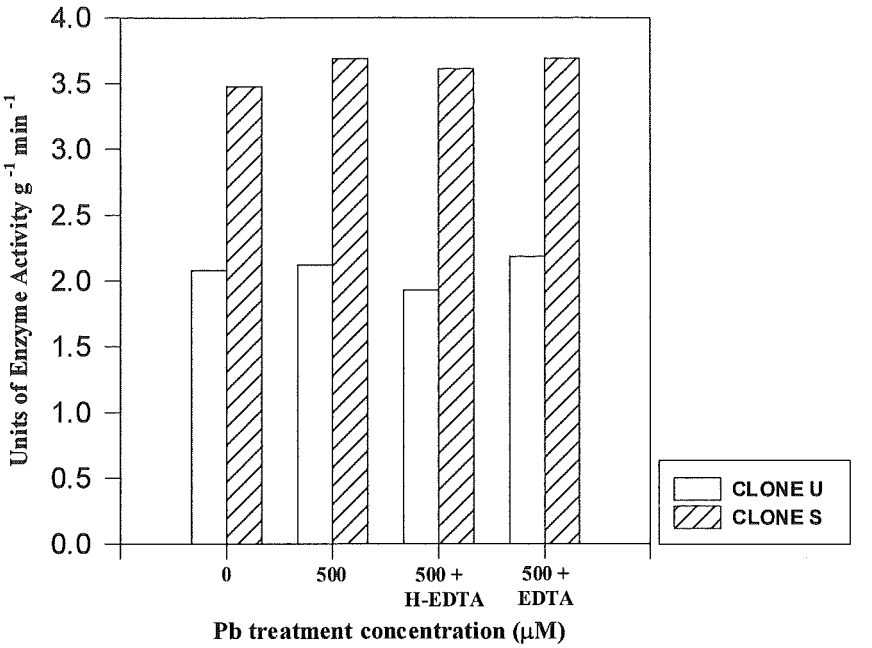


Figure 27a. Shoot acid phosphatase activity in extracts from 6 month-old *Pinus radiata* clones U and S, after 7 d in $Pb(NO_3)_2$ +/- 0.5 mM H-EDTA or EDTA.

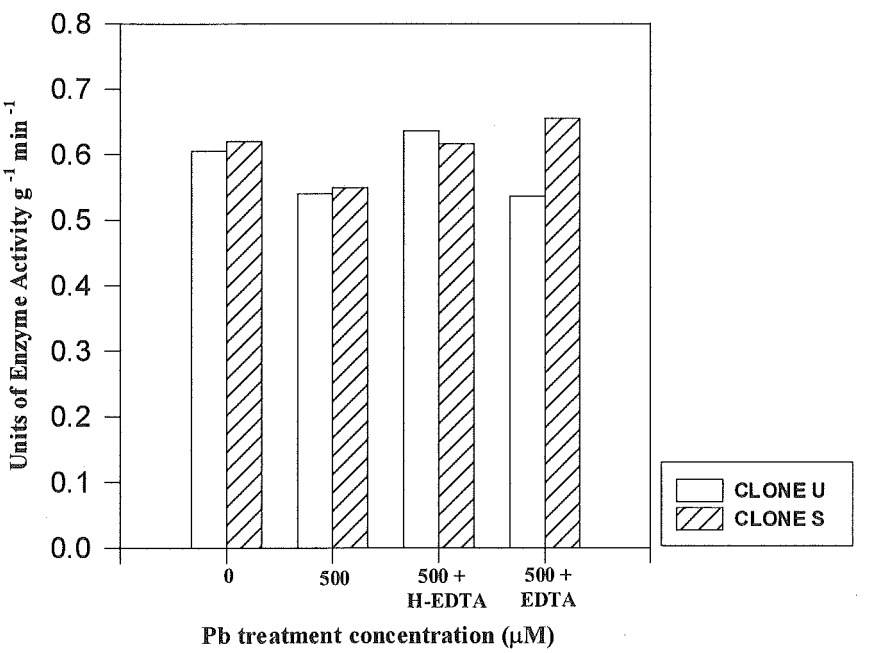


Figure 27b. Root acid phosphatase activity in extracts from 6 month-old *Pinus radiata* clones U and S, after 7 d in $Pb(NO_3)_2$ +/- 0.5 mM H-EDTA or EDTA.

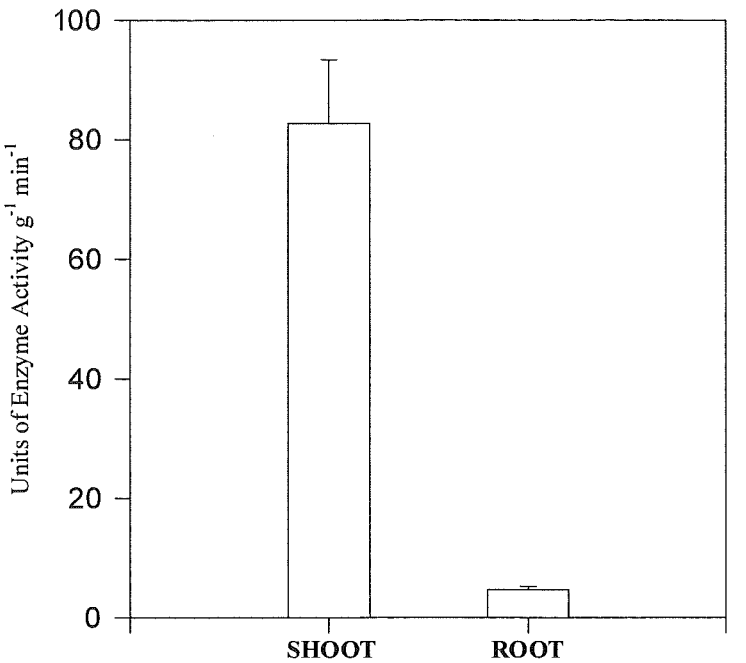


Figure 28. **Acid phosphatase activity in extracts from 12 week-old seedlings of *Chamaecytisus palmensis* after 6 days in a zero P environment. Bars represent standard error (n=3).**

When seedlings of *C. palmensis* were exposed to 500 μM Pb, with and without chelation at 0.125 mM for 7 days, shoot enzyme activity levels (Figure 29a) were more than 20 times higher than those of root (Figure 29b) in the same plants. Only one data point is represented at the H-EDTA treatment level for both root and shoot due to the poor condition of the plants in two of the rounds at the conclusion of the experiment.

When *C. palmensis* clone 7 was exposed to 500 μM Pb, with and without chelation at 0.125 mM for H-EDTA, or 0.5 mM for EDTA, for 7 days, shoot enzyme activity levels (Figure 30a) were approximately 15 times higher than root enzyme activity levels (Figure 30b) in the same plants. Shoot tissue exposed to EDTA had higher enzyme activity than shoot tissue exposed to H-EDTA but this was not the case for root tissue.

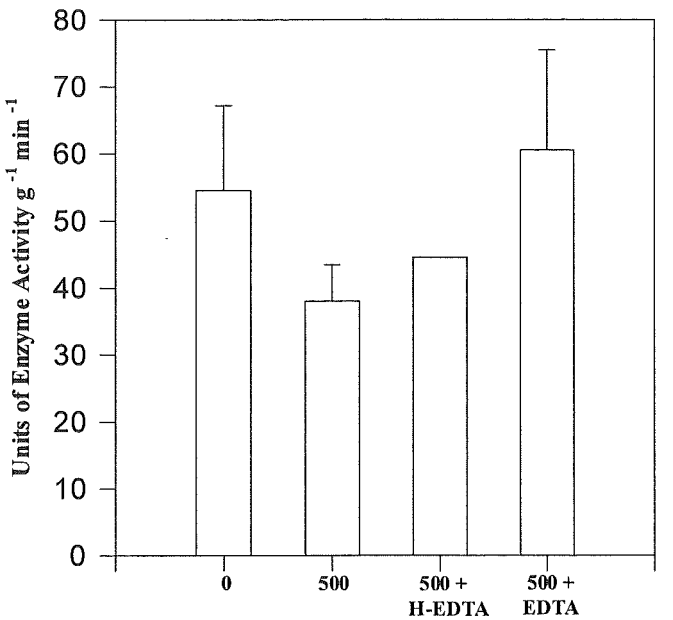


Figure 29a. Shoot acid phosphatase activity in extracts from 6 month-old seedlings of *Chamaecytisus palmensis* after 7 d in $Pb(NO_3)_2$ +/- 0.125 mM H-EDTA or EDTA. Bars represent standard error (n=1 or 3).

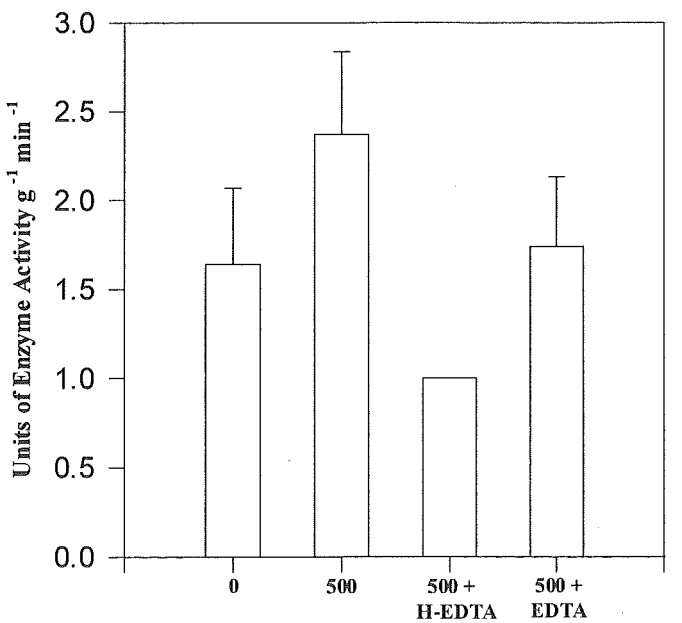


Figure 29b. Root acid phosphatase activity in extracts from 6 month-old seedlings of *Chamaecytisus palmensis* after 7 d in $Pb(NO_3)_2$ +/- 0.125 m H-EDTA or EDTA. Bars represent standard error (n=1 or 3).

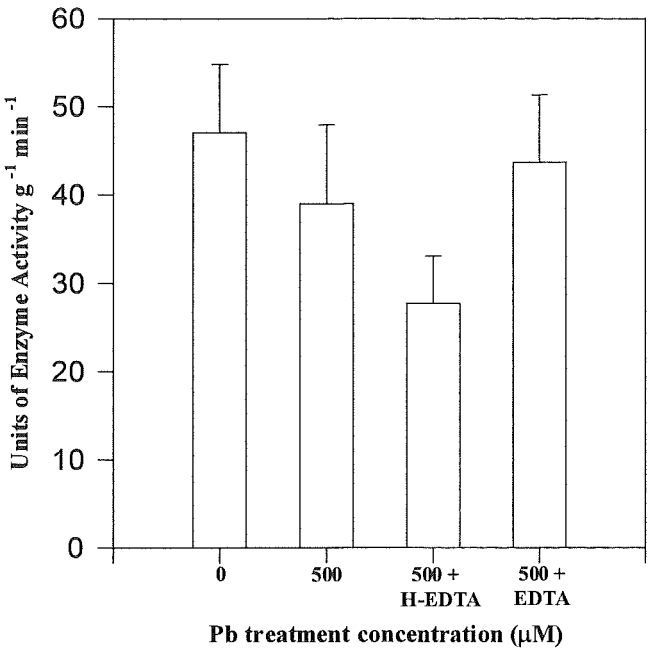


Figure 30a. Shoot acid phosphatase activity in extracts from 6 month-old *Chamaecytisus palmensis* clone 7 after 7 d in $Pb(NO_3)_2$ +/- 0.125 mM H-EDTA or 0.5 mM EDTA. Bars represent standard error (n=3).

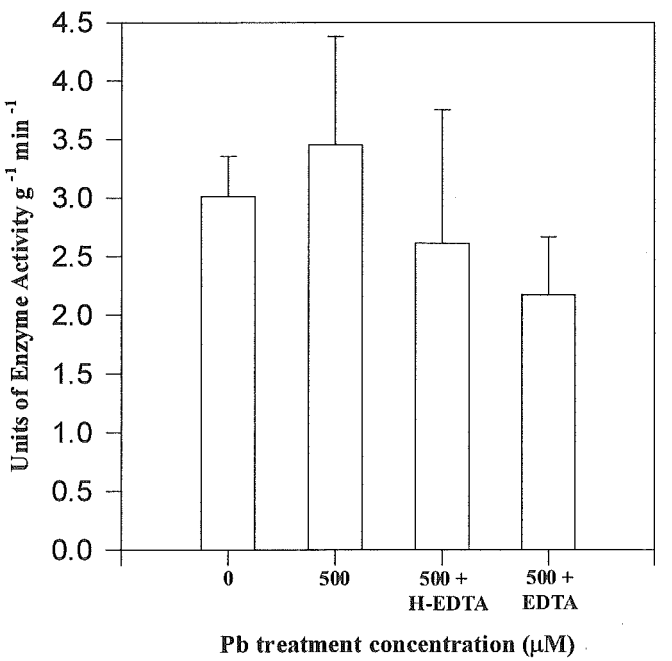


Figure 30b. Root acid phosphatase activity in extracts from 6 month-old *Chamaecytisus palmensis* clone 7, after 7 d in $Pb(NO_3)_2$ +/- 0.125 mM H-EDTA or 0.5 mM EDTA. Bars represent standard error (n=3).

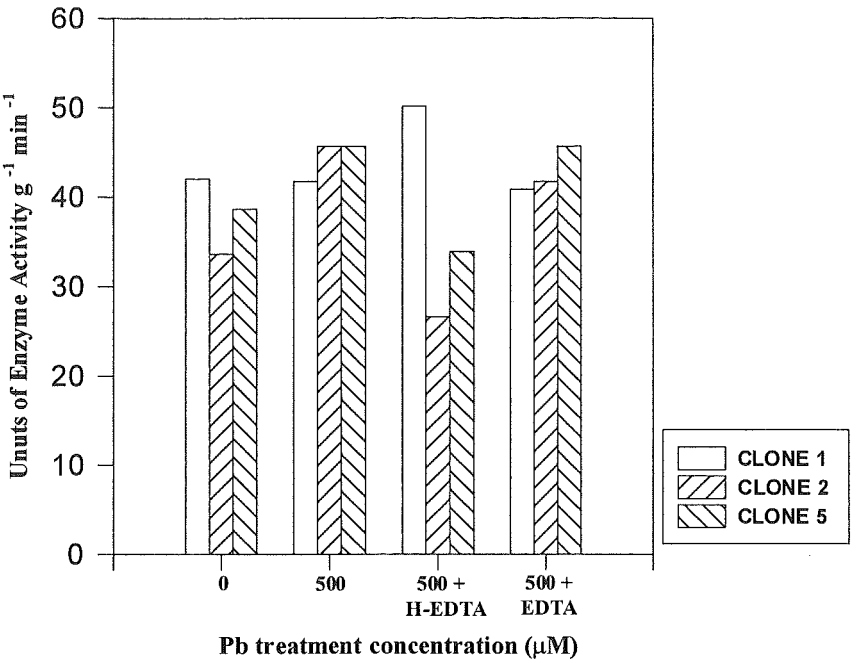


Figure 31a. Shoot acid phosphatase activity in extracts from 6 month-old *Chamaecytisus palmensis* clones 1, 2, and 5 after 7 d in $Pb(NO_3)_2$ +/- 0.125 mM H-EDTA or 0.5 mM EDTA (n=1).

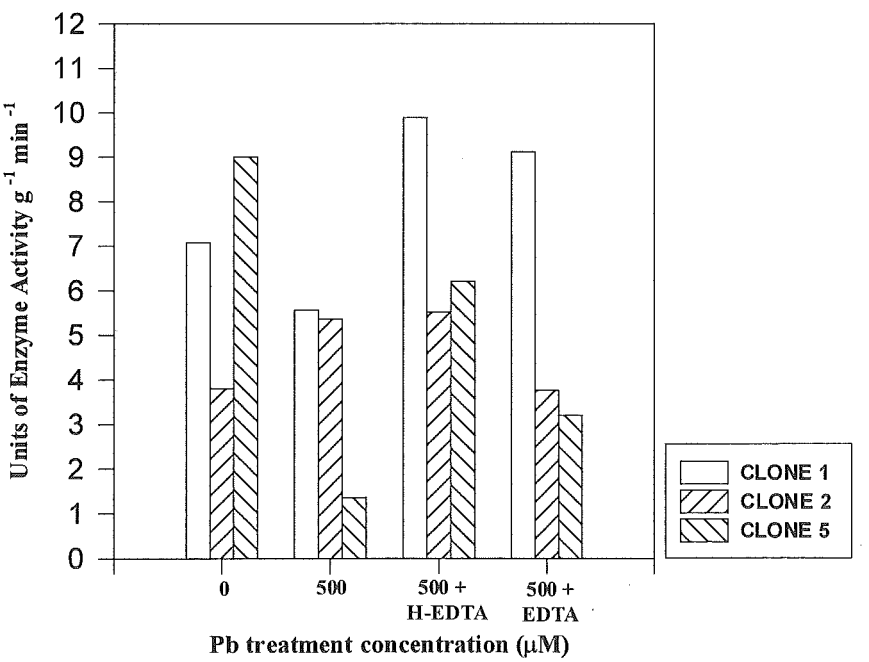


Figure 31b. Root acid phosphatase activity in extracts from 6 month-old *Chamaecytisus palmensis* clones 1, 2, and 5 after 7 d in $Pb(NO_3)_2$ +/- 0.125 mM H-EDTA or 0.5 mM EDTA (n=1).

C. palmensis clones 1, 2, and 5 were exposed to 500 μM Pb, with and without chelation at 0.125 mM for H-EDTA, or 0.5 mM for EDTA, for 7 days. Shoot enzyme activity levels (Figure 31a) were about 10 times higher than those of root, in the same plants (Figure 31b). Considerable variation was apparent within treatments and among clones for roots, and to a lesser degree for shoots, with no obvious pattern discernible.

6.2.1 Quantitative enzyme data : analysis of variance

Out of all analyses of treatment (factor 1), or clone (factor 2), only two P values with significance at the 99 % level or higher, were found:

(1) *P. radiata* shoot enzyme activity. Factor 'Clone' (Figure 26a).

$P = 0.0051$ **

(2) *P. radiata* shoot enzyme activity. Factor 'Clone' (Figure 27a).

$P = 0.00013$ ***

Unplanned multiple comparisons of means were performed on the data where significant P values were found, applying the Tukey test with the following results: (Means that are not significantly different are joined by a horizontal line, $\alpha = 0.05$).

(1) X W V T (Figure 26a)

(2) U S (Figure 27a)

6.2.2 Summary

Despite the variation observed in the results obtained from the quantitative enzyme experiments, certain trends were apparent. In general, shoot enzyme activity levels were higher than root enzyme activity levels, at nearly all levels of treatment, for both *P. radiata* and *C. palmensis.*

In *P. radiata* seedlings, a weak concentration-dependent effect of Pb on enzyme activity was observed between 250 μM Pb and 500 μM Pb, particularly with shoots (data not shown).

Although not found significant by anova, shoot tissue exposed to EDTA generally had higher levels of enzyme activity than comparable shoot tissue exposed to H-EDTA, for both *P. radiata* and *C. palmensis.*

6.3 Clonal variation in specific acid phosphatase activity

It was of interest to determine if there was clonal variation in acid phosphatase activity in *P. radiata* and *C. palmensis* in the different Pb treatments. First, total soluble protein determinations were carried out according to the method of Bradford (1976) on *P. radiata* clones T, and U, and *C. palmensis* clones 7, and 2. The results of these protein determinations are presented in table 22. These clones were chosen from preliminary experiments.

Table 22. Total soluble protein expressed in mg protein g^{-1} (f.w.), in clones of *Pinus radiata* and *Chamaecytisus palmensis,* after 7 days in 500 μM Pb, with and without chelation. The concentration of EDTA was 0.5 mM in all cases. The concentration of H-EDTA was 0.5 mM for *P. radiata* and 0.125 mM for *C. palmensis.* (Missing values were due to a shortage of clonal materials)

		P. radiata T	*P. radiata* U	*C. palmensis* 2	*C. palmensis* 7
ROOT	0 Pb	0.731	0.188	0.188	0.473
	500 μM Pb	1.065	0.273	0.396	0.254
	+ H-EDTA	0.877	0.315	-	0.269
	+ EDTA	0.931	0.600	0.362	0.119
SHOOT	0 Pb	2.392	2.177	1.562	1.881
	500 μM Pb	2.473	2.142	1.635	1.777
	+ H-EDTA	2.473	2.335	-	2.027
	+ EDTA	2.619	2.308	2.058	2.115

In table 23, the specific acid phosphatase enzyme activity of the clonal materials in different Pb treatments are presented. The root and shoot of clone U of *P. radiata* had higher specific acid phosphatase activity than those of clone T in all four Pb treatments. In contrast, substantial clonal variation was detected in the root only in the absence of both Pb and chelator when clone 2 was compared with clone 7 of *C. palmensis.*

Table 23. Specific acid phosphatase enzyme activity in extracts of roots and shoots of *Pinus radiata* clones T, and U, and *Chamaecytisus palmensis* clones 2, and 7 after seven days in 500 µM Pb, with and without chelation at 0.5 mM for EDTA in all cases, and 0.125 mM H-EDTA for *C. palmensis.* The enzyme activity is expressed as units per mg protein (Missing values due to shortage of clonal materials).

		P. radiata **T**	***P. radiata*** **U**	***C. palmensis*** **2**	***C. palmensis*** **7**
ROOT	0 Pb	0.291	3.218	20.21	6.36
	500 µM Pb	0.185	1.978	13.53	13.58
	+ H-EDTA	0.257	2.019	-	9.70
	+ EDTA	0.232	0.893	10.39	18.24
SHOOT	0 Pb	0.475	0.955	21.51	25.01
	500 µM Pb	0.636	0.989	27.91	21.95
	+ H-EDTA	0.471	0.826	-	13.68
	+ EDTA	0.462	0.948	20.27	20.61

(7) ISOZYMES OF ACID PHOSPHATASE

7.1 Preliminary trials

Initial trials aimed at refining the isozyme detection protocol were conducted on extracts from seedlings and clones, derived from fresh and frozen, Pb-exposed and non Pb-exposed *C. palmensis* root nodules, roots, and shoots.

In high pH native gels, in addition to unconcentrated extracts, both acetone precipitated and PEG dialysed extracts were run. In low pH native gels, acetone precipitated shoot extracts were run (see appendix H). Gels were incubated with stain for only 1.5-3 hours if sufficient band colour developed, otherwise they were incubated overnight.

Isozyme activity of extracts from *C. palmensis* seedlings and clones, was consistently detected in both high and low pH native gels. However, similar gel systems failed to detect phosphatase isozyme activity of extracts from *P. radiata* and further efforts with this species were discontinued due to time constraints.

7.2 High pH gels

Isozymes of acid phosphatase were detected in root nodules, roots, and shoots, of seedlings and clones of *C. palmensis,* in samples derived from fresh and frozen extracts. In root nodules, three different isozymes were detected at high pH, two with high electrophoretic mobility and one with low mobility. In roots, two different isozymes were always detected at high pH, one with low mobility and one with high mobility. On one occasion, at high pH, a third root isozyme was detected with intermediate mobility. When a boiled, concentrated sample derived from root extract, was included in a gel, it produced no activity, having been denatured, and helped to confirm that the bands produced by the unboiled samples represented enzyme activity, and were not merely artefacts. In shoots, one isozyme with high electrophoretic mobility was always detected at high pH (see appendix H).

Concentrated samples derived from thawed frozen root extracts of clone #7, after 7 days in 500 μM Pb, with and without chelation, were run on a high pH

gel (Plate 40). Prior to running the gel, each lane was loaded with equivalent quantities of total protein, relative to the most dilute extract, after Bradford analysis determined the total soluble protein concentration of each of the extracts. In each lane, one clear band of low mobility was produced. The extract from the 500 μM Pb treatment (lanes 4 & 5) produced greater intensity of enzyme activity per weight of total protein, than the other treatments. Due to the short duration of stain incubation, 2 hours, the band of high mobility was not clearly visible.

Pb treatment, in the presence or absence of a chelator, did not induce the synthesis of any novel acid phosphatase isozymes in *C. palmensis.* However, some evidence was found that Pb treatment alone could promote higher levels of enzyme activity.

PLATE 40 High pH native gel loaded with PEG-concentrated thawed frozen root extracts from *Chamaecytisus palmensis* clone #7 for acid phosphatase isozyme detection. Arrow indicates band of enzyme activity after staining at 37 ° C for 2 hours. All lanes loaded with equivalent quantities of protein.

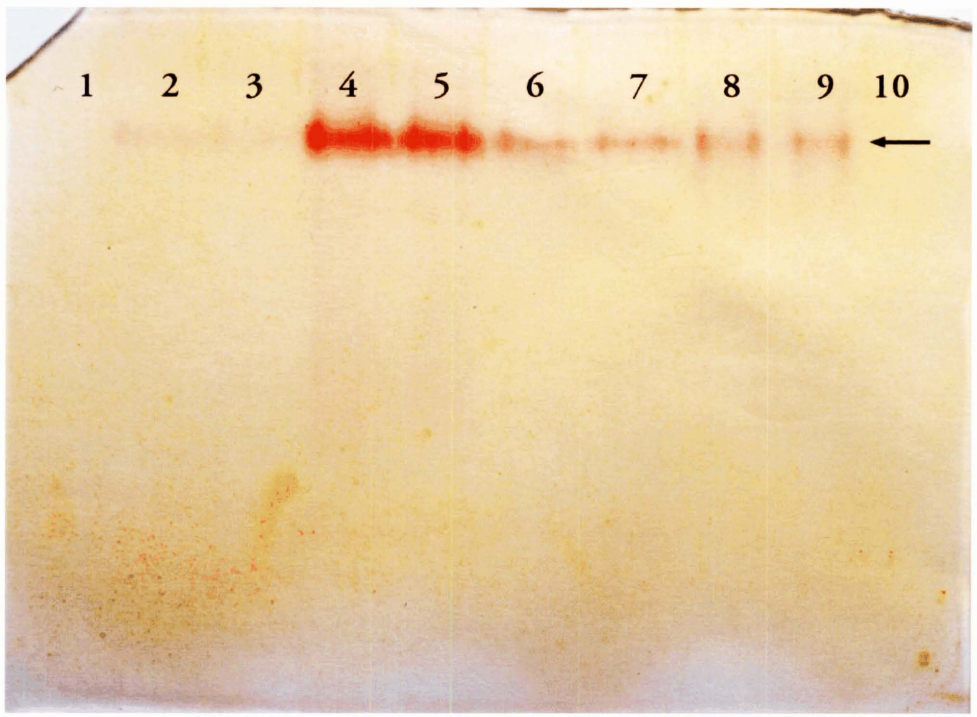
Lanes: 2 & 3 - non Pb-treated root.

4 & 5 - 500 μM Pb-treated root.

6 & 7 - 500 μM Pb + 0.5 mM H-EDTA treated root.

8 & 9 - 500 μM Pb + 0.5 mM EDTA treated root.

10 - dye marker.



High pH acid phosphatase isozyme native gel, run with frozen, concentrated root extracts from *Chamaecytisus palmensis* clone #7, after 7 days in 500 µM Pb with and without chelation. Equivalent quantities of protein were loaded in all lanes.

PLATE 40

CHAPTER IV

DISCUSSION

(1) TISSUE CULTURE OF C. PALMENSIS

There are few reports on any aspect of the culture of *Chamaecytisus palmensis* and most of those consider either its suitability as a fodder crop, among allied genera (Gonzalez-Andres and Ortiz, 1996), or aspects of its taxonomy and distribution (Sanchez-Yelamo *et al.*, 1995). Although regarded as having high potential for agroforestry systems of semiarid regions of the subtropics, to date, there are no reports on the *in vitro* culture and manipulation of this species.

Genetic manipulation of plants at the cellular and molecular levels for use in plant improvement programs, whether directly through gene transfer or indirectly through *in vitro* selection, requires the ability to regenerate whole plants from unorganised tissues (Han *et al.*, 1993). Regeneration of forest trees in general, and legumes in particular, through tissue culture has long been regarded as a difficult problem (Dwari and Chand, 1996). Other types of legumes including clovers and grain legumes, such as peas, have also proven difficult to regenerate from callus (Ezhova *et al.*, 1985; Schroeder et al., 1993; Grant *et al.*, 1995), leading some researchers to develop protocols for direct plant regeneration without an intervening callus phase (White and Voisey, 1994).

The ability to regenerate whole plants from tree legume-derived callus has been shown to be dependent on several factors such as explant origin, growth medium composition, choice of plant growth regulators, callus age, and genotype. In *Dalbergia lanceolaria* L., axenic seedling-derived cotyledon and hypocotyl explants from a 10 year old tree, displayed superior performance in callus induction, proliferation and regeneration compared with mature explants taken directly from the tree (Dwari and Chand, 1996). In contrast to the above study, callus initiation and subsequent shoot regeneration were obtained from cambial tissue of mature *Robinia pseudoacacia* L. (Han *et al.*, 1993). In that study, shoot regeneration was highly genotype specific and was only achieved with calli derived from one tree.

From the outset, the principal tissue culture goal in this study was the regeneration of whole *C. palmensis* plants from callus culture but it was understood that many potential difficulties could be encountered and that regeneration was by no means guaranteed. In light of this, a second major goal was identified, the production of clonal materials through the micropropagation of axenic seedling-derived materials.

1.1 Callus culture of *C. palmensis*

Often in tissue culture, callus can be initiated suitably, from appropriate explant materials, on media containing high auxin levels and low cytokinin levels, while shoot regeneration can be induced by transferring callus tissue to media containing low auxin levels and high cytokinin levels and this has been found to be the case with leguminous trees (Dwari and Chand, 1996; Han *et al.*, 1993), and non-leguminous trees (Lin and Griffin, 1992).

However, in the perennial shrub *Artemisia absinthium* L., 100% callus induction was achieved *in vitro* irrespective of the auxin:cytokinin ratios with the concentration of the cytokinin (BA) often exceeding that of the auxin (NAA, IAA). In addition, BA and 2,4-D alone, at higher concentrations, were each able to induce callus (Nin *et al.*, 1996).

Introduction of *C. palmensis* into callus culture was straightforward with callus development on all axenic seedling tissues (cotyledons, hypocotyls and roots) exposed to 10 μM 2,4-D. On 1/2 MS alone minimal callus development on cotyledons was achieved, although brown coloured and slower growing, as opposed to the vigorous, multi-coloured callus derived from 1/2 MS plus 2,4-D. Similarly whole immature embryos produced abundant callus/meristematic tissue after 10 days on 1/2 MS plus 10 μM 2,4-D. When uniform, mature, *C. palmensis* embryos were subjected to factorial combinations of auxin (2,4-D) and cytokinin (BA), callus development was evident at all levels, including controls, and no clear pattern emerged of explant response to plant growth regulator concentration. Weak evidence was found that higher cytokinin levels in combination with lower auxin levels were more effective at inducing callus. In contrast, when whole

immature *C. palmensis* embryos were subjected to a similar trial with factorial combinations of auxin (2,4-D) and cytokinin (BA), callus with a different, filamentous, morphology formed. Again, no clear pattern emerged of explant response to plant growth regulator concentrations but in this case it seemed that lower BA concentrations induced callus more effectively, independently of 2,4-D concentration. Eventually all callus induction trials were conducted with either cotyledons derived from mature seed, or whole immature embryos, as these types of explant proved to be the most responsive for callus initiation.

Transfer of callus from media containing auxin alone, or in combination with cytokinin, to media containing only cytokinin, a process sometimes referred to as reversal transfer, has been found to induce shoot regeneration in leguminous trees (Han *et al.*, 1993). When this was undertaken with *C. palmensis* callus, it did not induce organogenesis but the morphology of the callus usually altered, typically promoting changes in surface texture and colour. Often after transfer, the viability of cultures decreased but when callus was transferred to media containing TDZ and/or picloram, rapid growth was often seen for a limited period of time following the transfer.

It has been found that the morphogenetic potential of callus for regeneration decreases with both callus age and the number of culture passages. In the tree legume *Dalbergia lanceolaria* L., maximum shoot regeneration occurred from two week old callus derived from cotyledons and regenerative capacity decreased as the number of culture passages increased (Dwari and Chand, 1996).

When *C. palmensis* callus was subcultured, replicates gradually lost viability. Some cultures survived the transfer into a third subculture round but lost vigour from the outset and eventually died.

Callus formation alone however, is not sufficient to guarantee shoot regeneration. The morphology of the callus is extremely important and non-organogenic callus will probably never be coaxed into organogenesis. Often the most organogenic callus has a creamy-white colour, is friable and grows rapidly, such as that produced by *Dalbergia lanceolaria* L. (Dwari and Chand, 1996), where it retained its original colour, never developed green patches, nor turned compact or nodular in consistency, throughout organogenesis. In the regeneration study by Lin and Griffin (1992), on *Duboisia* hybrids, callus from shoot tips appeared white to slightly yellow, while callus from young seeds was white, loose

and proliferated very quickly on subculture. Not all organogenic callus needs to be creamy-white or yellow however, Han *et al* (1993), reported a requirement for the initiation and maintenance of green callus tissues in regeneration studies on seedling-derived cultures of *Robinia pseudoacacia* L.

Although a wide variety of callus morphologies was initiated from a variety of *C. palmensis* explant types, on media containing a wide range of plant growth regulators, a truly organogenic type of callus was not produced. Putative elements of a highly regenerative callus type, such as rapid growth, lack of nodularity, retention of looseness/friability, and white or creamy yellow colouration, were encountered separately in various callus cultures but never in combination. This does not necessarily mean however, that shoot regeneration will never be achieved from green *C. palmensis* callus tissue.

The great propensity which *C. palmensis* displays for callus initiation and growth to date, is matched closely by its recalcitrance to shoot regeneration. In light of the complexity and range of tissue culture protocols available to examine the potential *in vitro* responses of an uncharacterised species, it is realised that continued efforts will be required to achieve organogenesis. There are avenues for exploration that have not been fully explored, such as a more complete investigation into the response of callus tissue to photoperiod, light intensity, and perhaps even light quality. Also, there are explant sources such as vascular cambium (Han *et al.*, 1993), that have not been examined and which could potentially produce organogenic callus.

1.2 Micropropagation of *C. palmensis*

The multiplication of *in vitro* shoots proved to be uncomplicated with all clonal lines exhibiting equal ease of initial establishment upon transfer to fresh 1/2 MS media. Excised apical shoot sections from hyperhydrated clonal lines were no less amenable to transfer than the normal clonal line. However, after transfer of excised *in vitro* shoot sections, two main differences between the hyperhydrated and the normal clonal lines soon became evident. Firstly, the normal line, #7, always grew more vigorously, from the outset, than the hyperhydrated clones and this was almost certainly because roots formed spontaneously on every #7 shoot

within approx. 10 days of the transfer and when roots formed, concomitant rapid shoot growth ensued. When roots formed spontaneously on hyperhydrated shoots, which only happened sporadically and often took several weeks, these shoots also grew more vigorously than the un-rooted shoots, but not as vigorously as rooted shoots of clone #7. This was very likely due to the differences in root morphology that were evident between the hyperhydrated and normal clones. The highly vigorous, multi-branching roots of clone #7 were thicker, with an apparently greater surface area, and were more numerous than those that formed on the hyperhydrated clones. This would have allowed more effective water and nutrient uptake in the normal clones, leading to rapid, unimpeded shoot growth. In addition, it has been reported that in hyperhydrated cauliflower plants the vascular connection between the stem and the roots was incomplete, (Ziv, 1991).

The second major difference *in vitro* was the gradual colour change, after several weeks, from colourless and clear, to a translucent brown, of all growth media in which hyperhydrated shoots were growing. Over time, usually several months, the colour of the growth medium gradually darkened further. At no time did the growth media containing clone #7 change colour, even after many months.

It is possible that the wound response of the excised hyperhydrated clones includes the exudation of phenolic substances into the growth medium in such a way that wound callus formation is inhibited. High levels of phenols have been reported in hyperhydrated cultures of *Prunus avium*, (Ziv, 1991). When shoots of clone #7 are excised and transferred, the response is the formation of wound callus cells within days, rapidly followed by the differentiation of these cells into roots. This would help to explain why so few hyperhydrated shoots form roots, either sporadically, or when exposed to a root formation-inducing plant growth regulator, such as IBA. The continued release of phenolic substances into the growth medium, by hyperhydrated shoots, would also explain the increasing discolouration of the agar medium.

When rooted *in vitro* shoots were transferred to *ex vitro* conditions, normal shoots acclimatised more rapidly than hyperhydrated shoots. This is almost certainly due, at least in part, to the altered morphology of the epidermal tissue in the hyperhydrated shoots, which possibly lack elements of a cuticle and may also have decreased stomatal capabilities, factors which would contribute to the reduced ability of the shoots to tolerate conditions of lower humidity.

Leaf vitrescence associated with defective epidermal tissue caused by faulty deposition of epicuticular waxes has been reported in several species. Also, palisade tissue in hyperhydrated leaves has been found to be very thin, or lacking altogether and abnormal stomata that do not function properly and remain open in darkness or under conditions of water stress have been reported in several species, (Ziv, 1991).

As *C. palmensis* plants grow in *ex vitro* conditions, newly arising tissues gradually assume a 'normal' physiology and eventually the plants are visually indistinguishable from seed-raised individuals.

(2) LEAD EFFECTS AND UPTAKE

2.1 Lead effects

Changes in the growth patterns of roots and shoots of plants exposed to Pb in various forms, including $Pb(NO_3)_2$, have been reported in a number of studies (Przymusinski and Wozny, 1985; Breckle and Kahle, 1992; Huang and Cunningham, 1996; Sobotik *et al.*, 1998). These changes include inhibition of both root elongation and lateral root formation, changes in root morphology, root tip discolouration, and decreased root and shoot biomass accumulation. Often the magnitude of the effects on the plants was dependent on the Pb concentration.

In corn and ragweed, exposure to 20 μM Pb caused a reduction in root size after 5 days while at 100 μM Pb, root elongation and lateral root initiation ceased after 3-5 days, (Huang and Cunningham, 1996). In barley and maize seedlings, root growth was inhibited at lower Pb concentrations (1.0 mM) while shoot growth was unaffected. At higher Pb concentrations (10 mM), root growth was severely inhibited and shoot growth was reduced, (Sobotik *et al.*, 1998).

The concept that roots and shoots differ in their sensitivity to Pb has been reported elsewhere. Xiong (1998), concluded that in a cultivar of *Brassica pekinensis*, roots were more sensitive to Pb than shoots. In the same study it was also found that the inhibition of root and shoot length by Pb was concentration-dependent.

Exposure of *P. radiata* needles to high Pb levels by vacuum filtration (appendix I) demonstrated, albeit by an artificial method, the potential of these tissues for direct Pb uptake, rather than exclusion. Exposure of excised *P. radiata* shoots to high Pb levels in solution (appendix I), demonstrated some potential for Pb uptake by the vascular system.

Exposed to Pb in solution, at most concentrations, *P. radiata* seedlings displayed a range of responses including inhibition of root and shoot elongation, altered root morphology and colouration, inhibition of lateral root formation, and decreased biomass accumulation. At both high and low Pb concentrations, lateral root formation was severely inhibited, compared to the controls, while at the same time the treated roots changed to a dark brown colour compared to the white, pink, and light brown of the untreated roots. This discolouration may be due to precipitation of Pb on the root surface, perhaps in conjunction with phosphate containing molecules which have an affinity for Pb, or it may be due to cation exchange, where Pb ions may be displacing other divalent cations such as Ca^{2+} or Mg^{2+}. Pb is known to be effective at displacing various cationic metals from roots, (Harrison *et al.*, 1979).

As lateral roots are initiated within the pericycle, it may be postulated that exposure to Pb has interfered with the process whereby root primordia penetrate the cortex. It is thought that enzymes are involved in this process and a study of the distribution of β-glycerophosphatase in pea roots has shown that it is generally associated with cells about to die and undergo lysis, including cortical cells surrounding emerging lateral roots, (Bonnett, 1969). It is entirely possible that Pb ions compete or interfere with these enzymes preventing the nascent lateral roots from emerging. Alternatively, if Pb ions are able to penetrate the endodermis, the pericyclic divisions from which lateral roots arise may be interrupted altogether. In either case, the net result is a root system that is operating at reduced capacity with consequent decreased capability for water and nutrient uptake leading to diminished shoot growth and vigour.

Pb-treated *P. radiata* roots often became generally constricted and narrower, in some cases by up to 50 % for 2-3 mm, particularly at higher Pb concentrations. This finding is in contrast with that of Przymusinski and Wozny (1985), who found that the roots of young lupin seedlings exposed to high Pb

concentrations (4.5 mM), were much shorter and up to 3 times thicker than the controls.

P. radiata seedlings exposed to a range of Pb concentrations displayed concentration and time-dependent deleterious effects upon the health and vigour of the plants, over a six week period. At low Pb concentration, plants remained healthy longer than those at high Pb concentration, which displayed symptoms of stress after 1 week. It is probable that the decline in the vigour of the plants was due either directly to toxic effects induced by the cumulative uptake of Pb, or indirectly through the accumulation of Pb exposure-related effects such as depressed nitrogenase activity and/or inhibition of photosynthesis. Effects such as these were reported by Huang *et al.* (1974), in a study on the inhibition of soybean metabolism by Cd and Pb. If the effects on *P. radiata* were purely concentration dependent, then a more rapid decline would have been observed, particularly at the highest Pb concentration, instead of the gradual decline over time, that was recorded.

When the fresh weight changes, in *P. radiata* and *C. palmensis* seedlings exposed to a range of Pb concentrations, were measured after 14 days, it was found that marginally more biomass had accumulated at the 20 μM Pb level than in either the controls or at higher Pb concentrations, in both species. In the same experiment, when the lengths of the longest lateral roots were recorded, in *P. radiata* this occurred in 20 μM Pb, while in *C. palmensis* it was produced in the control.

Although it seems anomalous that Pb should be exerting any kind of growth promotion effect, at the very low concentration of 20 μM it is possible that the nitrate component may have been contributing enough of a nutrient effect to promote growth rates above that of the dH_2O controls. At Pb levels above 20 μM, the inhibitory effects of the Pb could possibly outweigh the nutritive effects of the nitrate. The concept of $Pb(NO_3)_2$ promoting growth has been described elsewhere.

Xiong (1998), reported that in *Brassica pekinensis*, compared to the control, total plant biomass tended to increase with increasing $Pb(NO_3)_2$ content in the growth medium, up to 500 $\mu g\ g^{-1}$, noting a combination of stimulatory and inhibitory effects. It was found that at 1000 $\mu g\ g^{-1}$, the inhibitory effect outweighed the stimulatory effect and the total plant biomass decreased but it was

still higher than in the control. On the basis of this, *B. pekinensis* was deemed to be a Pb-tolerant species.

Regarding lateral root length at the 20 μM Pb level, there may be a difference between the two species in Pb or nitrate sensitivity with regard to lateral root elongation. *P. radiata* may be insensitive to Pb and/or sensitive to nitrate while *C. palmensis* may be sensitive to Pb and/or insensitive to nitrate, at this low concentration.

Fresh weight determinations were made of excised *P. radiata* clonal shoots after 7 weeks exposure to a range of Pb concentrations *in vitro*. Although analysis of variance found that there was a more significant effect within clones, than within treatments, unplanned comparisons of means revealed that exposure to 200 μM Pb was significantly different to the control. In addition, the response to treatment, of clone #5, was found to be significantly different to the responses of clones #9, #4, and #2.

The ability of plant seeds to germinate and grow in the presence of Pb has been investigated by a number of researchers looking at various aspects such as; inhibition of rice seed germination (Mukherji and Maitra, 1977), ultrastructural localisation of Pb in germinating seeds of *Raphanus sativus* (Lane and Martin, 1982), seed germination and growth of the putative hyperaccumulator *Brassica pekinensis* (Xiong, 1998), and the extent to which seed coats are a barrier to Pb (Lane and Martin, 1977; Wierzbicka and Obidzinska, 1998).

Pinus radiata seeds stratified in dH_2O were transferred to solutions containing a range of Pb concentrations up to 1000 μM. As all of the seeds germinated successfully at every level of treatment, it can be said that *P. radiata* seeds are highly insensitive to Pb in the germination solution, at least up to 1000 μM. When *P. radiata* seeds were stratified in either 1000 μM Pb or dH_2O, then transferred to either 1000 μM Pb or dH_2O, in all cases the vast majority (> 80 %) of seeds germinated successfully. In many cases 100 % of the seeds germinated and in one case, seeds stratified in dH_2O and germinated in dH_2O scored only 90 %, indicating that lack of seed viability was possibly a factor in Pb treatments that scored less than 100 %. This finding indicates that *P. radiata* is insensitive to Pb in the stratification medium, at 1000 μM.

In a comparative study, where the germination and early seedling growth responses, to either dH_2O or 1000 μM Pb, of several species, including monocotyledons, dicotyledons and one gymnosperm were investigated, a diverse range of results was recorded. Lettuce, radish and corn were Pb-insensitive during germination but not during shoot and root elongation. Ryegrass was highly sensitive to Pb at all stages, wheat was highly Pb-sensitive to root elongation and clover was highly Pb-sensitive to germination and root elongation. Norway spruce was Pb-insensitive at all stages, notwithstanding the lack of shoot growth by this species after 7 days, in either 1000 μM Pb or dH_2O, as was tree lucerne which displayed marginally enhanced growth of roots and shoots, after exposure to Pb.

Radish, lettuce, and Norway spruce exhibited root tip discolouration after germination in 1000 μM Pb but only radish and lettuce displayed root elongation sensitivity thereafter. Root tip discolouration after exposure to Pb, may be due, in part, to the nature of the root cap, a thimble-like mass of cells that protects the apical meristem and which is morphologically and biochemically different to the rest of the root e.g., the slimy lubricating substance exuded by the root cap is a highly hydrated polysaccharide that is secreted by the outer root cap cells. Elements of root cap-cell physiology, such as longevity, are species specific and this suggests that other parameters, such as Pb-affinity, are also likely to vary from one species to another.

No clear pattern emerged of Pb sensitivity among the species tested, based upon classification by class (dicotyledones, monocotyledones), but the two conifers, *Picea abies* (Norway spruce) and *Pinus radiata* both demonstrated insensitivity to Pb during germination.

A key factor affecting sensitivity of seeds to Pb during germination is the extent to which the seed coat acts as a barrier. Lane and Martin, (1977) reported that the seed coat of *Raphanus sativus* acted as an effective barrier to Pb and helped prevent contamination of the embryo until the seed coat was torn apart by the germinating embryonic root. In contrast, Wierzbicka and Obidzinska (1998), found, in a study investigating differences in the structure of seed coats among 25 plant species in relation to the effect of Pb on imbibition and germination, that *R. sativus* had a seed coat that was very permeable to Pb. They found that among plants there are species with Pb-impermeable seed coats, semi-Pb permeable seed

coats and highly Pb permeable coats and that more than 70 % of the studied species had seed coats that were impermeable to Pb. The distinction was also made between Pb-permeability and water-permeability, highlighting the fact that although most species have water-permeable seed coats, so they can imbibe and germinate, this does not necessarily imply Pb-permeability.

In those species whose seed coats were Pb-permeable, Pb exposure delayed germination and lowered the ability of seeds to germinate in a dose-dependent manner. Similarly, a concentration-dependent effect of Pb upon the germination of rice (*Oryza sativa* L.) was reported by Mukherji and Maitra (1977), where the germination rate began to decline at a concentration of 0.02 M Pb acetate and was nil at 0.1 M.

The insensitivity to 1000 μM Pb, which both *C. palmensis* and *P. abies* exhibit with regard to germination and early seedling growth, may be linked to seed coat Pb impermeability. The insensitivity to a range of Pb concentrations, up to 1000 μM, which *P. radiata* exhibits with regard to germination may also be linked to seed coat Pb impermeability. It appears that *P. radiata* is moderately sensitive to Pb during early seedling growth and that despite its insensitivity to Pb during germination it is , at best, a marginally Pb-tolerant species. On the other hand, *C. palmensis* is Pb insensitive during germination and early seedling growth, and even displays marginally enhanced growth in the presence of Pb suggesting that it is a Pb-tolerant species.

2.2 Lead uptake

It was once believed that plants could take up and translocate only small amounts of Pb from soil and that when large quantities of Pb were found in the above ground portions of plants, it had originated from aerial sources, (Cannon and Bowles, 1962). This notion was overturned by Miller and Koeppe (1971), who demonstrated that corn plants could translocate and accumulate significant quantities of Pb in the leaves, in a concentration-dependent manner.

By the early 1980s, the general concept of phytoextraction had been established (Chaney, 1983) and since then, researchers have sought to define the

criteria by which plant's suitability for phytoextraction may be gauged, including factors such as tolerance to particular trace metals, volume of biomass produced, and most importantly, rates of metal accumulation.

It has been suggested that the goal of Pb phytoextraction is to reduce Pb levels in a soil to acceptable levels within a reasonable time frame (3-20 years), using plant species/cultivars that are able to accumulate greater than 1 % Pb (d.w.) in shoots (equivalent to 10 g kg^{-1}), and produce more than 20 t of biomass ha^{-1} $year^{-1}$ (Huang and Cunningham, 1996). However, by this definition, a species such as *Thlaspi rotundifolium*, which was reported to accumulate 1.1 % Pb (d.w.) on average (Reeves and Brooks, 1983), is unsuitable for Pb phytoextraction due to its slow growth and small biomass.

Both *P. radiata* and *C. palmensis* are species that produce significant biomass volumes. In New Zealand, *C. palmensis* is capable of producing up to 16 tonnes dry matter ha^{-1} in regions with approx. 700 mm annual rainfall (Snook, 1986). Although available yield figures for *P. radiata* in New Zealand, such as 670-800 m^3 ha^{-1} (Clifton, 1994), generally apply to harvestable timber volumes after 25-30 years, there is no doubt that it is a high-biomass producing species, particularly during the first 2 or 3 years of growth when it would be most suitable in phytoextraction.

The majority of research conducted in the field of Pb phytoextraction has been on known hyperaccumulators or their relatives, in particular, members of the Brassicaceae. The most widely studies species to date has been *Brassica juncea*, (Kumar *et al.*, 1995; Blaylock *et al.*, 1997; Vassil *et al.*, 1998). Other species which have also been characterised include corn and ragweed, (Huang and Cunningham, 1996), pea, corn, ragweed, goldenrod and sunflower,(Huang *et al.*, 1997), and *Brassica pekinensis*, (Xiong, 1998).

In the earliest Pb phytoextraction studies, some agronomic crops such as corn and pea were found to have the ability to accumulate shoot Pb to a concentration greater than *T. rotundifolium*. However, these crops could rarely accumulate shoot Pb concentrations greater than 1000 mg kg^{-1} (d.w.), which was far short of the 10,000 mg kg^{-1} (d.w.) target for Pb phytoextraction (Huang *et al.*, 1997). To increase Pb availability in soil, and promote Pb translocation from roots to shoots, the use of synthetic chelators, such as EDTA and H-EDTA, was introduced in Pb phytoextraction research and has increased the range of species

which may potentially serve as Pb phytoextractors. In recent times, most reports of Pb phytoextraction research, with very few exceptions, have included the use of synthetic chelators in one form or another. In fact, one of the two currently accepted strategies for phytoextraction, namely induced phytoextraction, relies heavily upon the use of synthetic chelators for effective soil remediation. In keeping with this trend, virtually all Pb uptake experiments conducted with both *P. radiata* and *C. palmensis* included the synthetic chelators H-EDTA and EDTA, at various concentrations.

A possible criticism of Pb phytoextraction research is that, in some cases, the studies may be unrealistic and not reflect conditions in the 'real world', particularly with regard to the high concentrations of Pb that are often used under experimental conditions. One answer to this critcism is that at higher treatment concentrations, analysis of plant materials is considerably more straightforward, and cost effective. Also, if a species demonstrates the ability to accumulate Pb at high concentrations, then it is entirely possible that it will accumulate Pb at the much lower concentrations that are likely to be found in the environment.

All Pb uptake determinations in this study were carried out using flame a.a. spectrometry and although this necessitated relatively large sample sizes, this was not necessarily an undesirable situation. It is reasonable to conclude that the larger the sample analysed, e.g., 1.0 g, the more accurate the result when extrapolating from the absorbance data to the final plant Pb uptake rate. Another way of putting this, is that in an ideal situation, the whole plant would be analysed. Simple tests such as analysing Pb-spiked *C. palmensis* seedling samples (appendix J), or *P. radiata* needles vacuum filtrated in Pb, demonstrated that the flame a.a. measurement technique was sufficiently sensitive to detect variation at the 10-20 ppm level.

When initial Pb-uptake trials were conducted in Pb-contaminated soil-less media, it was anticipated that adsorption/cation exchange of Pb ions could quite possibly lead to low uptake levels and ambiguous results but it was thought that it would be useful to compare this system with the hydroponic system that was eventually going to be utilised. In the event, measured Pb-uptake from soil-less media, by both *C. palmensis* and *P. radiata*, was so close to the limits of detection that soil-less media was abandoned in favour of the hydroponic system.

Notwithstanding variation in the magnitude of Pb uptake from one experiment to another, generally for both *P. radiata* and *C. palmensis*, root uptake of unchelated Pb greatly exceeded shoot uptake of Pb in the presence of chelators, after seven days. In addition, when the mean values of the ratio of root uptake to shoot uptake of unchelated Pb, at both the 500 μM and 250 μM levels (tables 11 & 12) are compared, i.e. approximately. 294 : approximately. 44 , the concentration dependent effect on uptake can clearly be seen.

Typically, the uptake of unchelated Pb by shoots was very low for both species, often at or near the limits of detection. Conversely, when shoot uptake of Pb in the presence of chelators was substantial, root uptake of Pb in the presence of chelators was often negligible, for both species, whether the treatment solutions were aerated or not, for both seedlings and clones. In some clones when the chelator concentration was low, e.g. 0.125 mM H-EDTA (figure 22a), root uptake of Pb from the chelated Pb treatment solution was surprisingly high. This may have been due to an excess of unchelated Pb in the solution being taken up by roots in a similar fashion to that in the unchelated Pb treatment solution. Also, some clonal variation was apparent in the shoot uptake of Pb in the presence of chelators by *C. palmensis* clone 5 compared to clones 1 and 2 (table 20).

The concept that most unchelated Pb taken up by plants remains in the roots is well documented, (Kumar *et al.*, 1995; Huang and Cunningham, 1996; Huang *et al.*, 1997; Blaylock *et al.*, 1997) and appears to apply to essentially all species tested to date. The propensity which roots have for accumulating Pb is governed in part by factors to do with both root physiology and Pb/soil chemistry. In soils with pH between 5.5 and 7.5, Pb solubility is controlled by phosphate or carbonate precipitates and very little is available to plants even if they have the genetic capacity to accumulate it. Extremes in soil pH above 7.5 or below 5.5 will either decrease or increase the solubility accordingly, (Blaylock *et al.*, 1997). A key factor in improving Pb phytoextraction therefore, is to increase and maintain Pb availability in the soil solution.

Pb in soils is classified as a soft Lewis acid which implies a strong covalent character to many of the ionic bonds it forms in soils and plants. Pb retention in the roots is based upon binding to ion exchangeable sites on the cell wall and extracellular precipitation, mainly in the form of Pb-carbonates deposited

in the cell wall. The addition of synthetic chelates, such as H-EDTA or EDTA, in combination with low pH, effectively prevents cell wall retention of Pb, making it available for translocation to shoots. In a study on EDTA in Pb transport, Vassil *et al.* (1998), found that EDTA appeared to chelate Pb outside the plant, after which the soluble Pb-EDTA complex was transported through the plant, via the xylem, and accumulated in the leaves.

When *C. palmensis* was exposed to H-EDTA and to a slightly lesser degree, EDTA, symptoms of phytotoxicity developed in a concentration dependent manner. Vassil *et al.* (1998), quantified phytotoxicity due to EDTA treatment in *Brassica juncea* by monitoring shoot desiccation and found that increasing concentrations of EDTA caused significant reductions in shoot water content. An exception to this was when treatment was with equimolar Pb and EDTA, suggesting that EDTA-induced foliar necrosis was attributable to the presence of free protonated EDTA (H-EDTA) in leaves. They predicted that uncoordinated EDTA would be available to bind various essential divalent cations such as Fe^{2+}, Zn^{2+}, and Cu^{2+}, disrupting the biochemistry of the leaf cells and ultimately causing cell death. It seems reasonable then, to conclude that these processes were taking place in the leaves of *C. palmensis* when exposed to the synthetic chelators, particularly H-EDTA, which always proved to be more phytotoxic than EDTA at the same concentration. This could explain why uptake of EDTA-chelated Pb in shoots often exceeded uptake of H-EDTA-chelated Pb.

In extreme cases this phytotoxicity could ultimately cause the death of the plant but Huang and Cunningham (1996), suggested that the death of a plant prior to harvest would not preclude successful Pb phytoextraction if substantial plant biomass had been established prior to the chelate treatment.

Ultimately, *C. palmensis* proved to be slightly more effective at taking up Pb, chelated or unchelated, into roots and shoots, than *P. radiata,* under similar experimental conditions. In seedlings and clones of both species, shoot uptake of chelated Pb was typically in the range of 0.07-0.3 % (w/w) which falls far short of the theoretical 1 % (w/w) goal set for effective Pb phytoextraction. However, root Pb uptake was much higher, in the region of 0.35-0.6 % (w/w) and it has been suggested previously that the complete removal of plant roots, although more expensive, may be a cost-effective alternative in phytoextraction generally, (Entry *et al*, 1993; Kumar *et al.*, 1995). These uptake values represent the ability of the

unimproved plants to accumulate Pb and it is entirely possible that the future application of screening/selection techniques will ultimately produce plants with a greatly enhanced capacity for phytoextraction of Pb.

Clonal variation in Pb uptake by *P. radiata* or *C. palmensis* was not found significant by anova in this study. Despite this, some encouraging indications of potential clonal differences in Pb uptake levels were found, particularly in *C. palmensis.*

(3) TRANSMISSION ELECTRON MICROSCOPY

Transmission electron microscopy has been used to localise deposits, and elucidate effects of trace metals, particularly Pb, in plants for many years. Early ultrastructural studies include those on *Pisum* chloroplasts (Sabnis *et al.*, 1969), *Avena* coleoptiles (Zegers *et al.*, 1976), seeds of *Raphanus sativus* (Lane and Martin, 1982), and *Populus* tissue-culture cells (Ksiazek *et al.*, 1984).

In a histochemical study of Pb uptake in radish, Lane and Martin (1977) reported that Pb transport in the root was possible either through the apoplast or the symplast. Since then many ultrastructural studies have found that Pb accumulates predominantly in root cell walls (Ksiazek, 1984; Qureshi *et al.*, 1986; Wierzbicka, 1995; Sobotik *et al.*, 1998; Wierzbicka, 1998), in intercellular spaces (Ksiazek, 1984), and to a lesser extent in vacuoles (Przymusinski and Wozny, 1985; Antosiewicz and Wierzbicka, 1999).

In ultra-thin sections of *P. radiata* roots, after seven days of Pb-treatment, unchelated Pb was never found in vacuoles, dictyosomes, or intercellular spaces but often found embedded in or adjacent to the cell wall, in many cases near plasmodesmata. Darkly stained tannin was found in vacuoles in both Pb-treated and non Pb-treated cells. By careful examination at both high and low magnification, it was possible to conclude that the tannin deposits were not Pb, mainly due to physical characteristics. Pb particles, particularly the larger ones, always had sharp, angular edges with a very grainy, crystalline appearance. Tannin deposits were typically globular with smooth, poorly defined edges and often appeared as aggregates or continuous deposits that lined the periphery of entire vacuoles.

In many cases the Pb particles were so large the plasmalemma became deformed to accommodate them between the protoplast and the cell wall. These types of infoldings have been described elsewhere as 'pinocytic' vesicles (Lane and Martin, 1972; Sharpe and Denny, 1976; Ksiazek *et al*, 1984). Malone *et al* (1974), described a situation where the Pb deposits in the spaces between the cell wall and the plasmalemma were caused by Pb-containing dictyosome vesicles migrating to the cell periphery, fusing with the plasmalemma, and discharging the Pb contents into the extra-cellular space. In *P. radiata* roots, evidence of this phenomenon was not observed and although it cannot be ruled out, the fact is that many Pb particles were much larger than any of the dictyosomes seen in the same ultra-thin sections.

Sometimes, particularly in close proximity to plasmodesmata, the larger Pb particles appeared to occupy much of the volume of the cell wall. In other regions, where the cell wall was much thicker and more substantial, smaller particles accumulated within the cell wall but towards its periphery. The deposition of these smaller Pb particles may have occurred through the action of pinocytotic vesicles. Where Pb has been deposited between the cell wall and the cell membrane, it is probable that it has been transported apoplastically but elsewhere, e.g., where Pb is deposited practically within a plasmodesma, it has probably been transported symplastically. This notion is supported by the findings of Ksiazek (1984), who found that in tissue-cultured cells, two simultaneous modes of Pb transport could not be ruled out and suggested that large Pb deposits were indicative of apoplastic transport.

If the compartmentalisation of symplastically transported Pb occurs through dictyosome-mediated exocytosis, it may happen relatively quickly (e.g. hours rather than days), after initial Pb exposure. In which case, Pb would not be detected in dictyosomes after seven days, at which time the compartmentalisation process would be long completed and ultra-thin sections prepared at that time would not reflect all of the dynamic events that had previously occurred. Weirzbicka (1995) reported that Pb uptake in *Allium* roots was very intense during the first 4 hours and was most intense during the first 15 minutes. It was further reported that during the first 24 hours of Pb incubation, a large amount of Pb was accumulated and inactivated in cell walls, vacuoles, and dictyosomal vesicles.

In *P. radiata*, Pb from chelation treatment was not detected in roots nor was unchelated Pb found in shoots, a finding that was supported by the flame a.a. Pb-uptake data obtained from similar samples. In ultra-thin *P. radiata* shoot sections, Pb chelated with H-EDTA was very difficult to detect and was only seen clearly at 120,000 X magnification, where it appeared as very fine grains dispersed in material adjacent to, but not embedded in the cell wall. Pb from EDTA-chelation treatment however, was clearly seen at much lower magnification as numerous fine grains predominantly deposited within an intercellular space, strongly suggesting apoplastic transport as the mechanism of Pb movement in this situation. According to Huang *et al.* (1997), EDTA is highly effective at increasing Pb transport into the xylem in corn plants and translocating it from roots to shoots. This however involves movement of the Pb-EDTA complex through the endodermis within the mature zone of the root before it can enter the xylem stream and therefore an element of symplastic transport must also be involved. Blaylock *et al.* (1997), suggested that EDTA may directly facilitate Pb movement through root cell membranes and extracellular spaces.

Within the endodermis, large Pb particles cannot easily cross the Casparian strip due to their size and charge characteristics but once they have formed a complex with a chelator such as EDTA, their solubility increases, the particle size decreases and they become partially 'invisible' to those processes that would normally prevent their unrestricted movement such as precipitation with phosphates or carbonates, or binding to cell walls through mechanisms such as cation exchange.

The mechanism by which solutes, having moved symplastically from root epidermal cells to the parenchyma cells of the vascular cylinder, enter the vessels or tracheids of the xylem, is postulated to be some type of highly selective active-carrier transport, as opposed to facilitated diffusion (Raven *et al.*, 1999). Although it is certain that this mechanism did not evolve to transport Pb, and that naked Pb particles attempting to utilise it would be impeded to some extent, it is likely that a Pb-chelate complex could effect passage through it more successfully.

To the best of my knowledge, there are no reports on any aspect of the uptake of Pb into the root nodules of leguminous plants. In ultra-thin sections of root nodules derived from Pb-treated *C. palmensis* plants, unchelated Pb was found mainly in cell walls, particularly around intercellular spaces, and also in

bacteroids and mitochondria. The diverse nature of this deposition suggests a mixture of both apoplastic and symplastic Pb transport. The heavy deposition of Pb around the intercellular space and within the cell walls is suggestive of apoplastic transport while the occurrence of Pb within structures in the cytoplasm indicates a symplastic interaction. Within root nodule cells, the membranes of both bacteroids and mitochondria seem to have an affinity for Pb, perhaps due to the presence of cation-exchangeable sites. Analogous to this was the suggestion by Sabnis *et al.* (1969), that Pb ions found in *Pisum* chloroplasts were chelated to negatively charged sites on the grana membranes.

In some *C. palmensis* root nodules, endodermal tissue was found where an exclusion effect had apparently operated on unchelated Pb, with respect to particle size. The putative function of the root nodule endodermis as a diffusion layer separating regions of different osmolarity (Vincent, 1974), was quite possibly involved in producing this effect. The suberin lamellae of the endodermis can be seen in plate 23, where the alternating light and dense bands have been interpreted as consisting of wax and suberin, respectively (Raven et al., 1999).

In root nodule cells exposed to Pb chelated with H-EDTA, Pb was found only in minute quantities, and in similar cells exposed to Pb chelated with EDTA, Pb was not found at all. This suggests that most or all of the chelated Pb had either not entered the root nodule, or had not remained there and had moved into the root tissue, or more likely, the xylem stream.

Heavy depositions of unchelated Pb detected in ultra-thin root sections of Pb-treated *C. palmensis* were found in middle lamellae, intercellular spaces, and the material adhering to the primary cell wall. Pb was almost completely lacking from the primary walls in all of these sections. This is almost certainly due to differences in the composition of the various plant cell wall components. The middle lamella, for instance, is composed mainly of pectic substances while the primary cell wall contains cellulose, hemicellulose and pectin. Pb is known to bind to pectin to a greater extent than hemicellulose, and only to a very small degree with other cell wall components (Weirzbicka, 1998). The material adhering to the primary cell wall, which often has significant quantities of Pb associated with it, may be comprised of pectin components such as galacturonic and glucuronic acids. When these acids dissociate, Pb ions have been found to compete for the negatively charged cation-exchangeable sites that arise, and

which are normally saturated with Ca^{2+} (Weirzbicka, 1998). This could explain why this type of material, which in some sections occurred in relatively large quantities, was always associated with heavy deposits of unchelated Pb.

In contrast to *P. radiata*, chelated Pb was found in ultra-thin root sections of Pb-treated *C. palmensis* but only in very small quantities which is in general agreement with the majority of the flame a.a. Pb-uptake data derived from similar samples. In the case of Pb chelated with H-EDTA, almost negligible quantities were found, associated with regions of the cell wall. Pb chelated with EDTA however, was found in small quantities but in significant places. Here, the majority of the Pb detected was found within mitochondria or what appeared to be amyloplasts. Within some of the putative amyloplasts, most of the Pb was closely associated with what appeared to be starch grains where it formed aggregates around their edges, presumably due to some sort of precipitation or charge reaction. In the case of the mitochondria, Pb was found only within their tissues and they appeared to be unaffected by its presence. As was possibly the case with root nodules, the Pb ions may have been attracted to sites on the root mitochondrial membranes by differences in charge.

One possible explanation for the presence of small quantities of Pb in the root samples exposed to Pb plus chelators, is that not all the Pb in the treatment solutions was converted to a Pb-chelate complex, due to insufficient chelator concentration. This would have meant that a small residual level of uncomplexed Pb was unable to enter the xylem stream and translocate to the shoots, and was therefore available to those root cell constituents with an affinity for it.

Pb was not detected in most ultra-thin sections taken from *C. palmensis* shoots exposed to unchelated Pb, where the dominant features were chloroplasts and the sections resembled those of the non-Pb treated controls. However, in other sections derived from similar material, Pb was found in chloroplasts in particular.

It is not clear how unchelated Pb was able to translocate to the shoots when the evidence in most cases suggests that unchelated Pb does not translocate easily but the Pb uptake data derived from flame a.a. on Pb-treated *C. palmensis* shoots does suggest that occasionally, for whatever reason, unchelated Pb will translocate to the shoots. One possible reason for this could be handling errors when transferring *C. palmensis* plants from soil-less media to hydroponic

solution. If roots were handled carelessly, there was potential to damage them, in which case unrestricted uptake from the treatment solution, by the xylem, could result. Although it is not possible to state categorically that this never happened, every effort was made to prevent it, and the consistency of the vast majority of the results reflect this. The possibility exists therefore, that within the genome of *C. palmensis* there is potential for the uptake and translocation to the shoots of unchelated Pb.

When ultra-thin sections, of *C. palmensis* shoots exposed to Pb chelated with H-EDTA, were examined at moderate magnification, very fine Pb grains were found in primary pit fields in thick-walled xylem cells. The Pb grains were predominantly associated with pit-closing membranes. These membranes are typically comprised of middle lamellae and thin layers of primary cell wall (Cutter, 1969). The presence of Pb chelated with H-EDTA, within xylem tissue, accords well with flame a.a. Pb-uptake data derived from similar shoot samples, and is also in agreement generally with the findings of other researchers, (Huang, 1996). It must be said however, that to date, there are few reports if any, of ultrastructural observations on chelate-induced shoot Pb translocation in any plant species.

In ultra-thin sections of *C. palmensis* shoots exposed to Pb chelated with EDTA, Pb was found only at very high magnification. The presence of Pb within the internal structure of chloroplasts suggests symplastic transport, as does the presence of Pb grains within plasmodesmata. In these sections, negligible quantities of Pb were seen in the middle lamella or other regions of the cell wall indicating that apoplastic transport was not a major factor in Pb deposition in this situation.

A potential criticism of the use of transmission electron microscopy for the localisation of Pb within plant tissues, is the possible dissolution and/or redistribution of Pb during the chemical preparation of tissues for ultrastructural observations. These issues have been addressed in studies such as that by Antosiewicz and Wierzbicka (1999), which found in a critical review of Pb loss during specimen preparation, that in Pb-treated *Allium* roots, on average, 96.2 % of the Pb remained in the tissues after preparation. Qureshi *et al.* (1986), compared the distribution of Pb in *Anthoxanthum odoratum* root tips after chemical preparation of tissues (glutaraldehyde and osmium tetroxide) and after

physical preparation of tissues (freeze drying). No difference was found in the distribution of Pb deposits following these two methods.

Although the results of the above studies relate specifically to root tissues of the species investigated, the general conclusion is that the levels of loss or redistribution of Pb during chemical preparation for EM are acceptably low and that within certain limits, results obtained from tissues prepared in this manner are meaningful.

(4) ACID PHOSPHATASE

Phosphorous is an essential inorganic plant nutrient which is absorbed principally as either $H_2PO_4^-$ or HPO_4^{2-}, contributing approx. 0.1-0.8 % (d.w.) of the total average plant biomass. It is required for the formation of the energy transfer compounds ATP and ADP, nucleic acids, several essential coenzymes and phospholipids. It is also involved in the phosphorylation of sugars (Raven *et al.*, 1999). A major component of the autotrophic capability of higher plants is the ability to 'mine' inorganic compounds, such as P, directly from the environment and under P-deficient conditions it has been found that tissue extracts from many plants have an increased acid phosphatase activity (Besford, 1979).

Reports of investigations into diverse aspects of plant acid phosphatase enzymes have been published for many years. Among these are the histochemical study of the distribution and activity of acid phosphatases in several plant species by Shaykh and Roberts (1974), the isolation and partial characterisation of cytoplasmic and wall-bound acid phosphatases from wheat roots (Hasegawa *et al.*, 1976), quantitative aspects of leaf acid phosphatase activity in tomato plants (Besford, 1979), and the molecular analysis of phosphate starvation stress in tomato and *Arabidopsis* (Goldstein *et al.*, 1989).

Researchers have investigated the responses of other plant enzymes to Pb exposure for many years with studies that include changes in the activities of the enzymes protease, α-amylase, RNase, and DNase in germinating rice (Mukherji and Maitra, 1976), changes in the activities of rice seedling catalase, peroxidase, IAA oxidase, IAA synthase, and ascorbic acid oxidase (Mukherji and Maitra,

1977), and changes in the activity of nitrate reductase in mungbeans (Singh *et al.*, 1997).

Studies that specifically deal with changes in acid phosphatase activity in response to Pb, include that by Lee *et al.* (1976), who found in a study of enzymes in soybean leaves that $Pb(NO_3)_2$ at 100 $\mu g\ ml^{-1}$ for 10 days stimulated acid phosphatase, peroxidase, malic dehydrogenase, and α-amylase but depressed levels of glutamine synthetase. Maier (1978), found that in Pb-treated young corn plants, levels of acid phosphatase activity increased in the leaves and decreased in the roots.

One of the problems in studying the effect of Pb on plants is the tendency for Pb and P to precipitate together in traditional solution culture at pH 6.0-7.0. At least two effects flow from this, one of which is the reduction of available P to the plant, the other is the prevention, to a greater or lesser degree, of the uptake of Pb by the plant. To avoid the latter, nutrient solutions utilised in Pb phytoextraction research generally contain very low levels of P (Huang and Cunningham, (1996). The concentration of $H_2PO_4^{2-}$ in the HC nutrient used in this study was only 0.01 mM and at this level, at pH 4.5, $Pb(NO_3)_2$ up to 500 μM dissolved in it almost completely. Pb levels higher than this, in HC nutrient at pH 4.5, produced increasing white, cloudy, precipitation, with increasing Pb concentration.

It was hypothesised therefore, that exogenously applied Pb in the nutrient medium could stimulate the production of increased levels of acid phosphatase within the plant, compared to those in a control solution. As previously stated, other researchers have found this effect in leaves, and the opposite in roots.

In a low or zero P environment, *P. radiata* appeared to have comparable, low levels of acid phosphatase activity, with roots marginally more active than shoots. Considering the acid phosphatase activity in *P. radiata* exposed to unchelated Pb, there was some evidence that in comparison to the control, in shoots it was slightly elevated and in roots it decreased slightly. This apparent decrease in root acid phosphatase activity may be due in part to that element of the P-deficiency response that has been reported to involve excretion of acid phosphatase enzymes by roots, into the rhizosphere (Goldstein *et al.*, 1989). This would explain why, in some species at least, the root assayable fraction seems

partially depleted even when total enzyme activity has increased, as indicated by shoot enzyme activity levels.

The data generated from exposure to chelated Pb is less easy to interpret, perhaps due to a lack of association between the chelated Pb and P. In which case, it should have led to acid phosphatase activity levels comparable to the control but does not seem to have done so. The situation was complicated further by at least one other factor. The very low levels of P in the nutrient solution may have led to a situation where all the plants were in partial P-deficit from the outset, were consequently already producing elevated levels of acid phosphatase activity, and were therefore less responsive to enzyme stimulation by external sources such as Pb exposure.

In a zero P environment, shoot acid phosphatase activity in *C. palmensis* appeared to be greatly elevated relative to that of the root. In fact, in all subsequent *C. palmensis* acid phosphatase activity determinations, shoot levels were higher than root levels by approximately an order of magnitude, or more. When shoot extracts required dilution, it was typically found that the diluted extract displayed higher levels of activity (v/v), than the undiluted extracts and this was postulated to be caused by the presence of enzyme inhibitory substances whose effect was reduced more than proportionately upon dilution.

Generally, much of the data generated displayed a great deal of variability making meaningful interpretation difficult. In some trials where *C. palmensis* was exposed to unchelated Pb, relative to the control, shoot acid phosphatase activity decreased while root acid phosphatase activity increased. This situation was the reverse of that seen in *P. radiata* but did not hold true for *C. palmensis* in every case. *C. palmensis* plants exposed to Pb chelated with H-EDTA exhibited decreased acid phosphatase activity relative to the control, for both roots and shoots in many cases. This may have been due to the indifferent health of the plants at the conclusion of the experiment, caused by their inability to tolerate H-EDTA, even at the reduced concentration of 0.125 mM. In most cases, plants exposed to Pb chelated with EDTA produced levels of acid phosphatase activity that were comparable to the control but again, much variation was encountered and consequently results were somewhat ambiguous.

As a representative sample, acid phosphatase activity was related to total soluble protein for *P. radiata* clones T and U, and *C. palmensis* clones 2 and 7.

For clones T,U, and 2, enzyme activity by roots exposed to unchelated Pb was lower than the control while that of shoots was higher than the control. In clone 7, the reverse was found, i.e. the enzyme activity by roots exposed to unchelated Pb was higher than the control while that of shoots was lower than the control. Overall greater variability was encountered in results from *C. palmensis* than from *P. radiata* which may be due to species specific factors or inherent experimental variability.

Analysis of variance found no significant effect due to treatment in any of the trials and only two significant effects due to clonal differences in *P. radiata.* As a result of this, not too much weight can be attached to these findings but they do indicate possible trends that may occur in some of the enzymatic responses of these two species to Pb exposure and also suggest possible future experiments.

4.1 Isozymes of acid phosphatase

Isozymes, or multiple molecular forms of enzymes, are enzymes that share a common substrate but differ in electrophoretic mobility. Originally defined as different variants of the same enzymes, having identical or similar functions, and present in the same individual (Markert and Moller, 1959). Since their discovery by Hunter and Markert in 1957, isozymes have played a key role in many branches of biology (Hunter and Markert, 1957). During the 1960s their importance for understanding gene action in development and differentiation was exploited in both animals and plants. A review of this early work was made by Scandalios on six different kinds of enzymes (Scandalios, 1969). For some of them he noted differences in different parts of the same plant: both presence versus absence, and quantitative differences in concentration.

There were two principal goals in this section of the work, the first was to determine if different isozymes of acid phosphatase could be detected in the tissues of *P. radiata* and/or *C. palmensis*. The second was to ascertain, in the event of achieving the first goal, if exposure to Pb, both unchelated and chelated, caused the appearance of novel isozymes of acid phosphatase, or quantitative differences in concentration.

In *P. radiata* acid phosphatase isozymes were not detected, which was not altogether a surprising result as conifers in general have a reputation for being

difficult to extract protein from due to the wide range of interfering substances such as tannins, phenolics, and phenoloxidases that are commonly found within their tissues. It is highly likely that isozymes of acid phosphatase exist in *P. radiata* tissue but a more precisely defined protocol, particularly with regard to extraction buffer composition, will need to be developed to reveal them.

In *C. palmensis*, acid phosphatase isozymes were detected consistently in high and low pH gels, in samples derived from fresh and frozen extracts suggesting that they are quite robust with respect to handling and storage. At high pH, three different isozymes were detected in root nodules, two different isozymes were consistently detected in roots, and one isozyme was detected in shoots. The possibility that the third isozyme found in root nodules may be derived from the root nodule bacteria, cannot be ruled out on the basis of the evidence presented here.

In root nodules and roots, the least and most mobile isozymes migrated similar distances, implying structural similarities. The appearance of a third novel root isozyme with intermediate mobility at high pH, in a seedling extract, may have conceivably been displaying inherent genetic variation present within the species, as it was not possible to reproduce it, despite repeated efforts under similar conditions. At low pH, two different acid phosphatase isozymes were consistently detected in shoot extracts, both with low electrophoretic mobility.

As higher electrophoretic mobility generally translates to smaller particle size, it can be postulated that the more mobile isozymes are smaller than the less mobile isozymes, not withstanding differences in charge.

Novel isozymes were not detected due to Pb treatment but in gels with equivalent quantities of protein loaded, unchelated Pb seemed to promote higher levels of enzyme activity. This accords well with the theory that Pb treatment lowers the level of available P, thus inducing elevated acid phosphatase enzyme activity.

With appropriate genetic analysis it is theoretically possible to determine if the multiple isozymes revealed within each tissue type are encoded by alternate alleles at a single locus, or are determined by a multilocus genotype.

CHAPTER V

(1) GENERAL DISCUSSION AND CONCLUSIONS

Phytoremediation is still an emerging technology, or to be strictly accurate, set of technologies, and in the coming years as it becomes more well-defined and better established, the potential benefits that it offers will be greatly enhanced. At present it is seen as an adjunct to those systems that are currently used to remediate polluted environments, rather than a replacement. Although it is certainly true that as the technology currently stands, the most heavily polluted substrates existing today cannot be effectively remediated through plant-based methods alone, this may not always be the case.

Phytoextraction is still in its infancy, relatively, and there is enormous scope for improvement in ways that have not been addressed to date. Many researchers feel that genetic engineering, and the transgenic plants that result from it, is an appropriate path to follow but there is a wealth of genetic diversity that already exists within all plants that has yet to be revealed. Indeed, there is still a great distrust on the part of the general public regarding the indiscriminate release of genetically modified organisms into the environment, no matter how noble the cause.

In assessing the suitability of *P. radiata* and *C. palmensis* as candidates for improvement in the ability to phytoextract Pb, a number of key issues had to be addressed. Principally among them was tolerance to the metal. Although it may theoretically be possible to extract trace metals with plants that are intolerant to them, it is bound to be more effective in the long run if the plants grow satisfactorily, especially with relatively long-lived plants such as trees. Closely related to tolerance is the issue of metal uptake, as some plants tolerate metals by excluding them.

P. radiata, and particularly *C. palmensis* were found to tolerate Pb to a reasonable degree, especially beyond the stage of early seedling growth. Uptake of unchelated Pb, although restricted to mainly the root tissues, was surprisingly effective in both species, although *C. palmensis* was again superior in its ability to

achieve this. Despite this, neither species fulfil the currently accepted criteria for hyperaccumulation which essentially is 1 % (d.w.) uptake of metal by shoots.

It is currently thought that the solubilisation of Pb in contaminated soils, through the addition of chelating agents, is an appropriate step in phytoextraction methodology, and it does have the effect of promoting translocation of the metal to the shoots but it seems ironic that the addition of a second batch of often hazardous chemicals, whose safety in the environment is questionable at best, is necessary to achieve the removal of the original contaminant.

The ability to translocate Pb to the shoots in *P. radiata* and *C. palmensis* was greatly enhanced by the addition of chelating agents to the hydroponic medium and in the context of most current research in this area, this response is typical of most species investigated so far. The use of chelating substances in laboratory-based research is practical and useful as it helps identify different mechanisms of deposition within a range of plant tissues. It would however, be more ecologically sound if the use of these types of substances did not ultimately become widespread in the environment. If plants can't ultimately be 'encouraged' to translocate metals from the roots to the shoots, and root uptake levels are significant, then the removal of the whole plant, rather than just the shoots, would seem to be the logical answer. This concept has been largely ignored so far but it may gain acceptance over time.

In many respects the responses of *P. radiata* and *C. palmensis* to Pb, with respect to intra- and intercellular deposition and compartmentalisation, show similarities to the responses of most other species reported to date but in *C. palmensis* in particular, the extensive uptake of Pb by root nodules was novel and surprising.

The scope for improving the ability of *C. palmensis* in phytoextraction is high despite the lack of success in achieving shoot organogenesis. Its amenability to tissue culture manipulations and its wide range of responses to plant growth regulators suggest that in time, a regeneration protocol will be established. This would greatly facilitate *in vitro* selection opportunities but even as it stands, screening of *in vitro* clonal material for Pb tolerance will produce positive results given enough time. The same is true of *P. radiata,* a species that is regarded generally as recalcitrant in callus culture, but which may also be amenable to *in vitro* screening for Pb tolerance among clonal materials. This would be even more

effective if it can be demonstrated, as some researchers believe, that somaclonal variation acts not just upon de-differentiated tissue *in vitro*, but upon most plant tissue, most of the time.

It has been stated that in the wild, plants do not exhibit symptoms of toxicity to Pb even when it is present in high concentrations and this may be due to the unavailability of Pb due to co-precipitation with P, or other substances.

The link between Pb levels and the availability of P proved to be a more complex issue than was originally envisaged and although it was not fully elucidated through the measurement of acid phosphatase activity levels, some clues as to the nature of the relationship were found. Particularly the notion that root enzyme levels often decrease in response to Pb addition, while shoot enzyme levels increase. This phenomenon requires further study.

The inability to detect acid phosphatase isozymes in *P. radiata* was disappointing but, on the other hand the results of this section on *C. palmensis* were very encouraging, with isozymes revealed in all tissues. Particularly interesting was the appearance of novel bands, suggesting potential inherent molecular mechanisms within the species which could possibly translate to a greater ability to deal with situations of compromised P availability, such as exposure to Pb.

(1.1) Future work

The main thrust of future work in association with this study would be undertaking *in vitro* selection trials on *C. palmensis* for tolerance to Pb. Efforts to achieve shoot regeneration would be an integral part of this, but in the event that regeneration proved unattainable, selection protocols could be established on *in vitro* shoot material. These types of studies could include characterisation of clonal materials at the genetic level. In addition, a more comprehensive ultrastructural study could be undertaken on chelate-induced shoot Pb translocation in general as this is poorly understood at present and inadequately represented in the literature. More detailed studies on acid phosphatase activity levels in response to Pb exposure and isozymes found in *C. palmensis* could also be undertaken.

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APPENDIX A

Modified Quoirin and LePoivre Nutrient Medium (LP)

LP Major Stock Solution (20X)	1 Litre	2 Litres
KNO_3	36.0 g	72.0 g
$Ca(NO_3)_2.4H_2O$	24.0 g	48.0 g
NH_4NO_3	8.0 g	16.0 g
$MgSO_4.7H_2O$	7.2 g	14.4 g
KH_2PO_4	5.4 g	10.8 g

LP Minor and Vitamin (1000X)	500 ml	1 Litre
$ZnSO_4.7H_2O$	4.3 g	8.6 g
H_3BO_3	3.1 g	6.2 g
$MnSO_4.4H_2O$	10.0 g	20.0 g
$CuSO_4.5H_2O$	0.125 g	0.25 g
KI	0.04 g	0.08 g
$Na_2MoO_4.2H_2O$	0.125 g	0.25 g
$CoCl_2.6H_2O$	0.0125 g	0.025 g
Thiamine HCl	0.20 g	0.40 g

Fe Stock Solution (50X)	250 ml	500 ml
$FeSO_4.7H_2O$	0.375 g	0.75 g
Na_2EDTA	0.5 g	1.0 g

Nutrient Solution (Full Strength)	1 Litre
Major salts stock (20 X)	50.0 ml
Minor salts stock (1000 X)	10.0 ml
Fe stock (50 X)	20.0 ml
Myo-inositol	1.0 g
Sucrose	30.0 g
Agar	8.0 g

APPENDIX B

Huang & Cunningham Nutrient Solution

Major Salts Stock (10 X)		1 Litre	2 Litres
KNO_3	(2.0 mM)	2.022 g	4.044 g
$Ca(NO_3)_2$	(0.5 mM)	1.181 g	2.360 g
$MgSO_4$	(0.2 mM)	0.493 g	0.986 g
NH_4NO_3	(0.1 mM)	0.0808 g	0.1616 g
KH_2PO_4	(0.01 mM)	0.0136 g	0.0272 g

Minor Salts Stock (100 X)		500 mL	1 Litre
KCl	(50 μM)	0.1864 g	0.3727 g
FeEDTA	(20 μM)	0.36705 g	0.7341 g
H_3BO_3	(12 μM)	0.0371 g	0.07421 g
$MnSO_4$	(2.0 μM)	0.0223 g	0.0446 g
$ZnSO_4$	(0.5 μM)	0.0072 g	0.0144 g
$CuSO_4$	(0.2 μM)	0.00249 g	0.00499 g
Na_2MoO_4	(0.1 μM)	0.00121 g	0.00242 g
$NiSO_4$	(0.1 μM)	0.00078 g	0.00155 g

Nutrient Solution (Full Strength)	500 mL	1000 mL	2000 mL
Major Salts Stock (10 X)	50 mL	100 mL	200 mL
Minor Salts Stock (100 X)	5 mL	10 mL	20 mL
dH_2O	→500mL	→1 L	→2 L
Adjust pH to 4.5-5.0.			

NOTE: This nutrient solution has been formulated specifically for use in Pb uptake experiments. It has a low pH and P concentrations set at 10 μM or lower to ensure maximum Pb solubility/availability in solution.

(Huang & Cunningham, 1996)

APPENDIX C

Hoaglands Modified Nutrient Solution

Major Salts Stock		Concentration	Grams 100 ml^{-1}
(A)	$Ca(NO_3)_2 . 4H_2O$	1.0 M	23.61
(B)	KNO_3	1.0 M	10.11
(C)	$MgSO_4 . 7H_2O$	1.0 M	24.64
(D)	KH_2PO_4	1.0 M	13.61

Minor Salts Stock		
$MnCl_2 . 4H_2O$	-----	0.181
H_3BO_3	-----	0.286
$ZnSO_4 . 7H_2O$	-----	0.022
$CuSO_4 . 5H_2O$	-----	0.008
$H_2MoO_4 . H_2O$	-----	0.009

Iron Stock		
$Na_2FeEDTA$	------	3.67

Nutrient Solution (Full Strength)		500 ml	1000 ml	2000 ml
Major salts stock	(A)	2.5 ml	5.0 ml	10.0 ml
	(B)	2.5 ml	5.0 ml	10.0 ml
	(C)	1.0 ml	2.0 ml	4.0 ml
	(D)	0.5 ml	1.0 ml	2.0 ml
Minor salts stock		0.5 ml	1.0 ml	2.0 ml
Iron stock		0.5 ml	1.0 ml	2.0 ml

APPENDIX D

CITRATE BUFFER (0.1 M pH 5.0)

Stock solution (A)	0.1 M citric acid	2.101 g in 100 ml dH_2O
Stock solution (B)	0.1 M sodium citrate	2.941 g in 100 ml dH_2O

Full strength: 20.5 ml (A) + 29.5 ml (B) made up to 100 ml with dH_2O

(G. Gomori, 1955).

APPENDIX E

Anova tables for Pb-uptake data subjected to analysis of variance.

(fig. 11 & 12) **ROOT**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Treatment	12.954	3	4.318	62.491	1.35E-07	3.490
Round	1.577	4	0.394	5.706	0.0083	3.259
Error	0.829	12	0.069			
Total	15.360	19				

(fig. 11 & 12) **SHOOT**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Treatment	11.576	3	3.859	14.573	0.00026	3.490
Round	2.970	4	0.742	2.804	0.07433	3.259
Error	3.177	12	0.265			
Total	17.723	19				

(fig. 11 & 12) **R/S**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Treatment	28.045	3	9.348	22.52645	3.22E-05	3.4903
Round	1.117	4	0.279	0.673028	0.623275	3.25916
Error	4.980	12	0.415			
Total	34.142	19				

(fig. 13) **ROOT**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Treatment	3.378	3	1.126	10.350	0.009	4.757
Round	0.097	2	0.049	0.446	0.660	5.143
Error	0.653	6	0.109			
Total	4.128	11				

(fig. 13) **SHOOT**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Treatment	4.563	3	1.521	95.499	1.87E-05	4.757
Round	0.809	2	0.405	25.402	0.001	5.143
Error	0.096	6	0.016			
Total	5.468	11				

(Appendix E cont.)

(fig. 13) **R/S**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Treatment	10.541	3	3.514	23.878	0.0010	4.757
Round	0.622	2	0.311	2.113	0.202	5.143
Error	0.883	6	0.147			
Total	12.046	11				

(fig. 14) **ROOT**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Treatment	5.633	3	1.878	32.308	0.0004	4.757
Round	0.053	2	0.027	0.460	0.652	5.143
Error	0.349	6	0.058			
Total	6.035	11				

(fig. 14) **SHOOT**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Treatment	2.163	3	0.721	0.846	0.517	4.757
Round	0.219	2	0.109	0.128	0.882	5.143
Error	5.115	6	0.853			
Total	7.497	11				

(fig. 14) **R/S**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Treatment	7.491	3	2.497	4.930	0.047	4.757
Round	0.414	2	0.207	0.408	0.682	5.143
Error	3.039	6	0.507			
Total	10.944	11				

(fig. 16) **ROOT**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Treatment	2.802	3	0.934	11.811	0.006	4.757
Round	0.784	2	0.392	4.956	0.054	5.143
Error	0.475	6	0.079			
Total	4.061	11				

(Appendix E cont.)

(fig. 16) **SHOOT**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Treatment	1.135	3	0.378	7.350	0.020	4.757
Round	0.939	2	0.469	9.122	0.015	5.143
Error	0.309	6	0.051			
Total	2.382	11				

(fig. 16) **R/S**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Treatment	0.872	3	0.291	6.315	0.028	4.757
Round	0.109	2	0.054	1.180	0.370	5.143
Error	0.276	6	0.046			
Total	1.256	11				

(fig. 17) **ROOT**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Treatment	3.602	3	1.201	14.526	0.004	4.757
Round	0.586	2	0.293	3.547	0.096	5.143
Error	0.496	6	0.083			
Total	4.684	11				

(fig. 17) **SHOOT**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Treatment	5.453	3	1.818	81.024	3.03E-05	4.757
Round	0.068	2	0.034	1.527	0.291	5.143
Error	0.135	6	0.022			
Total	5.656	11				

(fig. 17) **R/S**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Treatment	12.001	3	4.000	76.289	3.61E-05	4.757
Round	0.257	2	0.129	2.453	0.167	5.143
Error	0.315	6	0.052			
Total	12.573	11				

(Appendix E cont.)

(fig. 18) **ROOT**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Treatment	3.071	3	1.024	31.502	0.0005	4.757
Round	0.053	2	0.026	0.813	0.487	5.143
Error	0.195	6	0.032			
Total	3.319	11				

(fig. 18) **SHOOT**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Treatment	3.108	3	1.036	22.944	0.0011	4.757
Round	0.105	2	0.052	1.161	0.375	5.143
Error	0.271	6	0.045			
Total	3.483	11				

(fig. 18) **R/S**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Treatment	8.992	3	2.997	51.395	0.0001	4.757
Round	0.304	2	0.152	2.603	0.153	5.143
Error	0.350	6	0.058			
Total	9.646	11				

(fig. 19) **ROOT**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Treatment	4.336	3	1.445	129.659	0.001	9.277
Round	0.000	1	0.000	0.029	0.876	10.128
Error	0.033	3	0.011			
Total	4.370	7				

(fig. 19) **SHOOT**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Treatment	2.558	3	0.853	10.747	0.041	9.277
Round	0.013	1	0.013	0.158	0.717	10.128
Error	0.238	3	0.079			
Total	2.808	7				

(Appendix E cont.)

(fig. 19) **R/S**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Treatment	9.875	3	3.292	32.460	0.009	9.277
Round	0.009	1	0.009	0.087	0.787	10.128
Error	0.304	3	0.101			
Total	10.189	7				

(fig. 20) **ROOT**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Treatment	6.432	3	2.144	46.287	8.54E-06	3.863
Clones	0.164	3	0.055	1.180	0.371	3.863
Error	0.417	9	0.046			
Total	7.012	15				

(fig. 20) **SHOOT**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Treatment	3.520	3	1.173	28.460	6.34E-05	3.863
Clones	0.040	3	0.013	0.320	0.811	3.863
Error	0.371	9	0.041			
Total	3.930	15				

(fig. 20) **R/S**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Treatment	12.042	3	4.014	85.507	6.2E-07	3.863
Clones	0.292	3	0.097	2.076	0.174	3.863
Error	0.422	9	0.047			
Total	12.757	15				

(fig. 21) **ROOT**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Treatment	4.665	3	1.555	19.122	0.00179	4.757
Round	0.188	2	0.094	1.159	0.375	5.143
Error	0.488	6	0.081			
Total	5.342	11				

(Appendix E cont.)

(fig. 21) **SHOOT**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Treatment	4.486	3	1.495	16.831	0.003	4.757
Round	0.008	2	0.004	0.045	0.956	5.143
Error	0.533	6	0.089			
Total	5.027	11				

(fig. 21) **R/S**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Treatment	8.560	3	2.853	15.689	0.003	4.757
Round	0.185	2	0.093	0.509	0.625	5.143
Error	1.091	6	0.182			
Total	9.836	11				

(fig. 22) **ROOT**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Treatment	2.760	3	0.920	6.299	0.028	4.757
Clones	0.518	2	0.259	1.773	0.248	5.143
Error	0.876	6	0.146			
Total	4.154	11				

(fig. 22) **SHOOT**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Treatment	4.304	3	1.435	58.038	7.98E-05	4.757
Clones	0.071	2	0.035	1.430	0.311	5.143
Error	0.148	6	0.025			
Total	4.523	11				

(fig. 22) **R/S**

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Treatment	8.198	3	2.733	15.570	0.003	4.757
Clones	0.307	2	0.154	0.875	0.464	5.143
Error	1.053	6	0.176			
Total	9.559	11				

APPENDIX F

Pb uptake data: unplanned comparisons of treatment means

For seedlings and clones, all means of log 10 transformed treatments (factor 1) were compared within root, shoot, and the root:shoot ratio. For clones, all means of log 10 transformed clones (factor 2) were compared within root, shoot, and the root:shoot ratio. All means are ranked in order of magnitude, those that are not significantly different from each other are joined by a horizontal line (Table 1).

Table 1. Unplanned multiple comparisons of means as determined by the Tukey test for equal sample sizes ($\alpha = 0.05$).

Fig. 11 & 12: Root 0 H E 500 Shoot 0 500 H E R:S E H 0 500	Fig. 13: Root 0 H E 500 Shoot 0 500 H E R:S E H 0 500
Fig. 14: Root 0 H E 500 Shoot 0 500 H E R:S H E 0 500	Fig. 16: Root 0 H E 500 Shoot 0 500 H E R:S 0 E H 500
Fig. 17: Root 0 H E 500 Shoot 0 500 E H R:S H E 0 500	Fig. 18: Root 0 E H 250 Shoot 0 250 H E R:S E H 0 250
Fig. 19: Root 0 H E 500 Shoot 500 0 E H R:S H E 0 500	
Fig. 20: Root 0 H E 500 Shoot 0 500 H E R:S H E 0 500	Fig. 20: Root V X W T (clones) Shoot W T V X R:S V X T W
Fig. 21: Root 0 E H 500 Shoot 0 500 H E R:S E H 0 500	
Fig. 22: Root E 0 500 H Shoot 500 0 H E R:S E H 0 500	Fig. 22: Root 2 5 1 (clones) Shoot 2 5 1 R:S 2 1 5

APPENDIX G

ACID PHOSPHATASE ASSAY: QUANTITATIVE CALCULATIONS

After all absorbance readings were recorded, compensated absorbance values were plotted to ensure linearity. Compensated values were obtained by subtracting the control absorbance value from the absorbance value given by the total ingredients. The middle value of the three data points generated for each level of treatment was chosen as being representative of enzyme activity for that sample. These values were initially converted to total activity values, then adjusted for weight, time, and if required, dilution factor, to ultimately provide values in units of enzyme activity g^{-1} (f.w.) min^{-1}.

EXAMPLE:

total plant weight = 0.25 g

total buffer volume = 5.0 ml

after spinning = 4.2 ml

adjusted buffer volume = 5.0 ml

volume for assay = 250 µL

assay duration = 5 min

compensated absorbance value = Y OD units (Optical Density) (eg 0.030)

TOTAL ACTIVITY = $\frac{\text{adjusted buffer volume x Y}}{\text{assay volume}}$

= $\frac{5.0 \text{ ml x } 0.030}{0.250 \text{ ml}}$

= 20 x 0.030

= 0.6 units of enzyme activity

ADJUSTING FOR WEIGHT: 0.6 / 0.25 = 2.4 units of enzyme activity g^{-1}

ADJUSTING FOR TIME: 2.4 / 5 = 0.48 units of enzyme activity g^{-1} min^{-1}

(appendix G cont.)

DILUTION FACTOR: $Z = \frac{\text{total diluted volume x Y OD}}{\text{assay volume}}$

(eg 1 : 6)

(0.214 ml : 1.286 ml)

Total diluted volume = 1.5 ml

Assay volume = 100 µL

$= \text{eg } \frac{1.5 \text{ ml x } 0.030}{0.10 \text{ ml}}$

$= 0.45$

TOTAL ACTIVITY (WITH DILUTION) $= \frac{\text{Total buffer volume x Z}}{\text{diluted enzyme volume}}$

$= (5.0 \times 0.45) / 0.214$ ml

$= 10.5$ units of enzyme activity

ADJUSTING FOR WEIGHT: $10.5 / 0.25 = 42$ units of enzyme activity g^{-}

ADJUSTING FOR TIME: $42 / 5 = 8.4$ units of enzyme activity g^{-1} min^{-1}

APPENDIX H

Acid phosphatase isozyme gel electrophoresis

When extracts of fresh *C. palmensis* root or shoot material were electrophoresed at high pH, distinct bands of staining, indicating acid phosphatase activity, were evident on the gel (plate 41a). The root extracts, particularly the concentrated ones, displayed two discrete regions of staining, indicating the presence of two different acid phosphatase isozymes, one with low electrophoretic mobility and one with high electrophoretic mobility. The shoot extracts had only one band, similar to the highly mobile root band.

When thawed frozen seedling root or shoot extracts, were run at high pH, intensity of enzyme activity similar to that of the fresh extracts was observed (plate 41b). This gel included a boiled, concentrated sample derived from root extract (lane 6), which having been denatured, produced no activity and helped to confirm that the bands produced by the unboiled samples represented enzyme activity, and were not merely artefacts. In this gel, the concentrated root sample (lane 4), produced a third, intermediate band, of relatively low mobility, that was not seen previously. The shoot samples each produced only one band, equivalent to the most mobile band produced by each root sample.

When concentrated samples, derived from fresh or thawed frozen seedling root extracts, were run at high pH, very similar banding patterns occurred right across the gel (plate 42a). Only two bands were observed for each sample: an intensely stained low mobility band, and a weakly stained highly mobile band.

In a gel where all samples were concentrated and derived from fresh root extracts, fresh root nodule extracts, or thawed frozen root extracts, novel bands of enzyme activity were observed (plate 42b). The root nodule samples each produced three bands of enzyme activity; one of low mobility (equivalent to the low-mobility root band), and two bands of high mobility (one of which was equivalent to the high-mobility root band, the other being even more mobile).

Acetone-precipitated samples derived from fresh shoot extracts of clone #7, after 7 days in 500 μM Pb, with and without chelation, were run on a high pH gel (Plate 43). In addition, acetone-precipitated samples derived from thawed frozen seedling shoot extracts were included.

(Appendix H cont.)

The pattern of band formation was very similar right across the gel with one band of high mobility produced by each sample, irrespective of origin.

Low pH gels

Acetone precipitated samples derived from fresh seedling shoot extracts were run on a low pH gel (Plate 44a). In the low pH gel system, regions of enzyme activity had a tendency to stain quite diffusely, complicating the interpretation of results. Despite this, it is reasonably clear that in plate 44a at least two bands of low mobility are apparent.

Another commonly occurring problem in low pH gels, evident after completion of staining, was uneven running of samples. Typically, the clearest results were obtained from the lanes in the centre of the gel as those at either side often appeared to have 'lagged' behind. To prove that this phenomenon was due to attributes of the gel, and not of the loaded samples, a low pH gel was run with nearly all lanes containing identical samples (Plate 44b). Six of the ten lanes contained acetone precipitated samples derived from thawed frozen shoot extracts of clone #7 after 7 days in 500 μM Pb plus 0.125 mM H-EDTA. Two bands of activity are apparent but not evenly across the gel and it appears that some of the samples have been 'reluctant' to run into the gel. The clearest bands are those on lane 5 (0 Pb), where the intense staining possibly indicates elevated enzyme activity.

The problem of uneven running can be seen even more clearly in plate 45a. This low pH gel was loaded with acetone precipitated samples derived from fresh or thawed frozen shoot extracts of clone #7, after 7 days in 500 μM Pb, with and without chelation. In each of lanes 3-6 (frozen extracts), two bands of low mobility are visible whereas in lanes 7-10 (fresh extracts), very little activity can be discerned (Plate 45a).

Similarly, in another low pH gel where the samples were all acetone precipitated and were derived from fresh shoot extracts of clone #2, and thawed frozen shoot extracts of clone #7, uneven running of the gel has partially obscured the results (Plate 45b). In several lanes two distinct low mobility bands are evident but the lanes in the middle of the gel provide the clearest results.

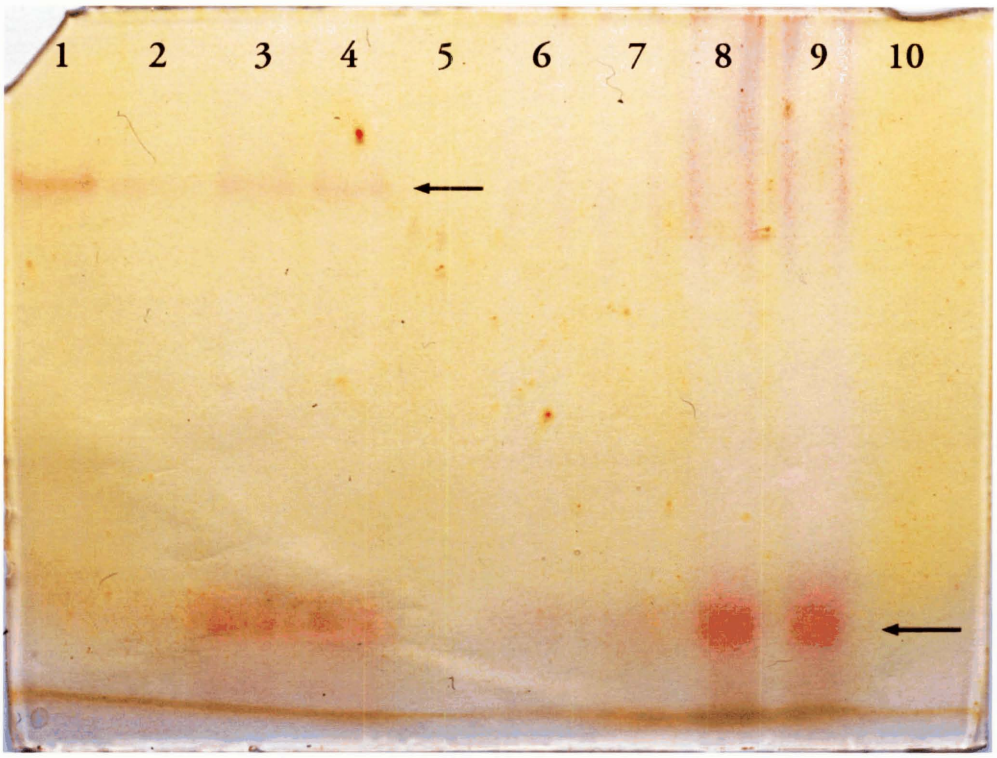
PLATE 41 High pH native gels loaded with root and shoot extracts from *Chamaecytisus palmensis* seedlings for acid phosphatase isozyme detection. Similarly labeled lanes contain replicate extracts from the same source.

(A) Gel run with fresh extracts. Arrows indicate bands of enzyme activity after staining at 37° C for approx. 15 hours.

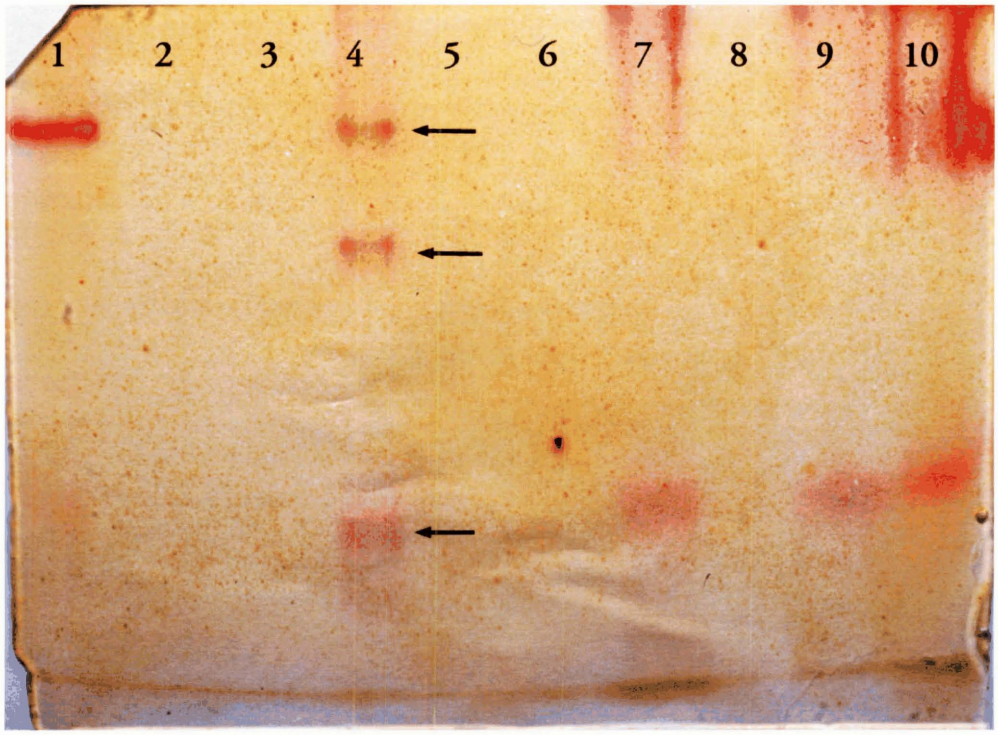
Lanes: 1 & 2 - unconcentrated root (100μL).
3 & 4 - concentrated root (100μL).
5 - dye marker (100μL).
6 & 7 - unconcentrated shoot (100μL).
8 & 9 - concentrated shoot (100μL).

(B) Gel run with thawed frozen extracts. Arrows indicate bands of enzyme activity after staining at 37 ° C for approx. 15 hours.

Lanes: 1 - unconcentrated root (100μL).
4 - concentrated root (100μL).
6 - boiled concentrated root (100μL).
7 - unconcentrated shoot (100μL).
8 - dye marker (60μL).
9 & 10 - concentrated shoot (50μL).



(A) High pH acid phosphatase isozyme native gel, run with fresh root and shoot extracts from *Chamaecytisus palmensis* seedlings



(B) High pH acid phosphatase isozyme native gel, run with frozen root and shoot extracts from *Chamaecytisus palmensis* seedlings.

PLATE 41

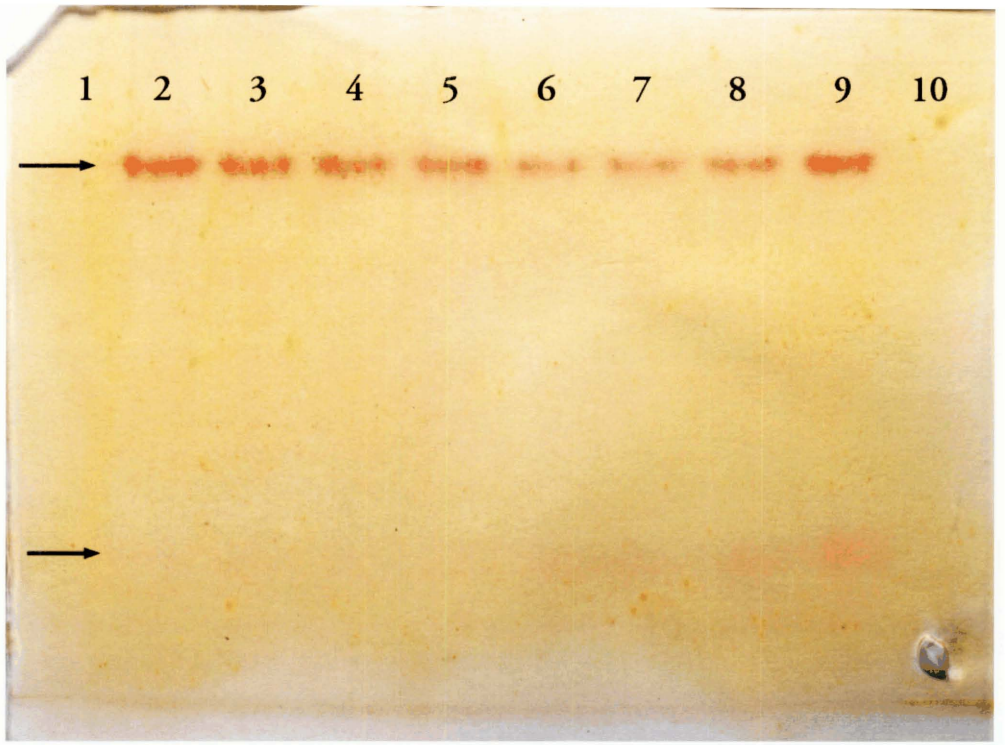
PLATE 42 High pH native gels loaded with root and root nodule extracts from *Chamaecytisus palmensis* seedlings for acid phosphatase isozyme detection. Similarly labeled lanes contain replicate extracts from the same source.

(A) Gel run with fresh and thawed frozen extracts. Arrows indicate bands of enzyme activity after staining at 37 ° C for approx. 15 hours.

Lanes: 1 - dye marker (10μL).
2-5 - concentrated fresh root (60μL).
6-9 - concentrated thawed frozen root (60μL).
10 - dye marker (10μL).

(B) Gel run with fresh and thawed frozen extracts. Arrows indicate bands of enzyme activity after staining at 37 ° C for approx. 15 hours.

Lanes: 1 - dye marker (20μL).
2-4 - concentrated fresh root nodule (100μL).
5-7 - concentrated thawed frozen root (100μL).
8-10 - concentrated fresh root (100μL).



(A) High pH acid phosphatase isozyme native gel, run with fresh and frozen concentrated root extracts from *Chamaecytisus palmensis* seedlings.

(B) High pH acid phosphatase isozyme native gel, run with frozen and unfrozen concentrated root and root nodule extracts from *Chamaecytisus palmensis* seedlings.

PLATE 42

PLATE 43 High pH native gel loaded with shoot extracts from *Chamaecytisus palmensis* clone #7 for acid phosphatase isozyme detection. Similarly labeled lanes contain replicate extracts from the same source. All lanes loaded with 60 µL of acetone-precipitated extract. Arrow indicates band of enzyme activity after staining at 37° for approx. 15 hours.

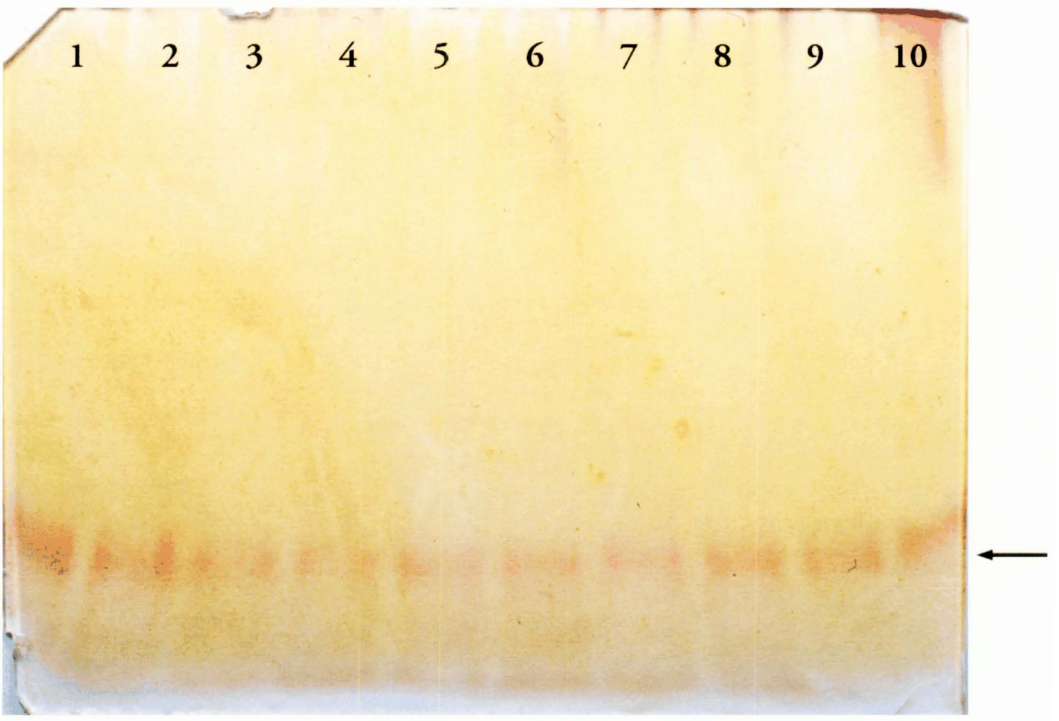
Lanes: 1 & 2 - thawed frozen non Pb-treated shoot.

3 & 4 - fresh non Pb-treated shoot.

5 & 6 - fresh 500 µM Pb-treated shoot.

7 & 8 - fresh 500 µM Pb + 0.5 mM H-EDTA treated shoot.

9 & 10 - fresh 500 µM Pb + 0.5 mM EDTA treated shoot.



High pH acid phosphatase isozyme native gel, run with fresh and frozen concentrated shoot extracts from *Chamaecytisus palmensis* clone #7, after 7 days in 500 µM Pb, with and without chelation.

PLATE 43

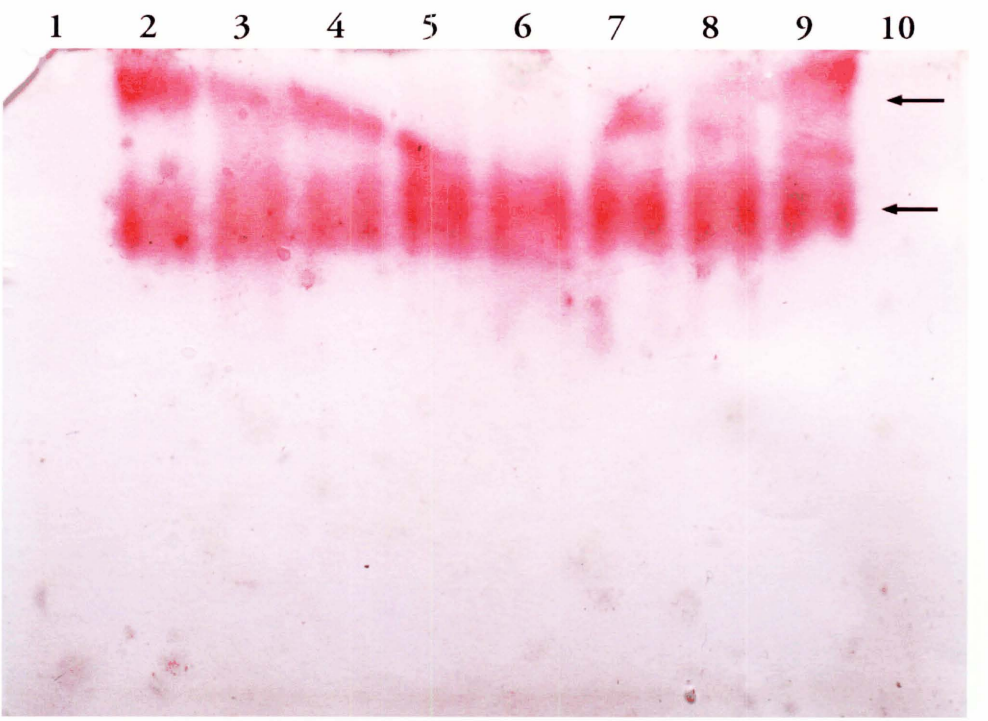
PLATE 44 Low pH native gels loaded with shoot extracts from *Chamaecytisus palmensis* seedlings and clone #7 for acid phosphatase isozyme detection. Similarly labeled lanes contain replicate extracts from the same source.

(A) Gel run with acetone-precipitated fresh seedling extracts. Arrows indicate bands of enzyme activity after staining at 37 ° C for approx. 15 hours.

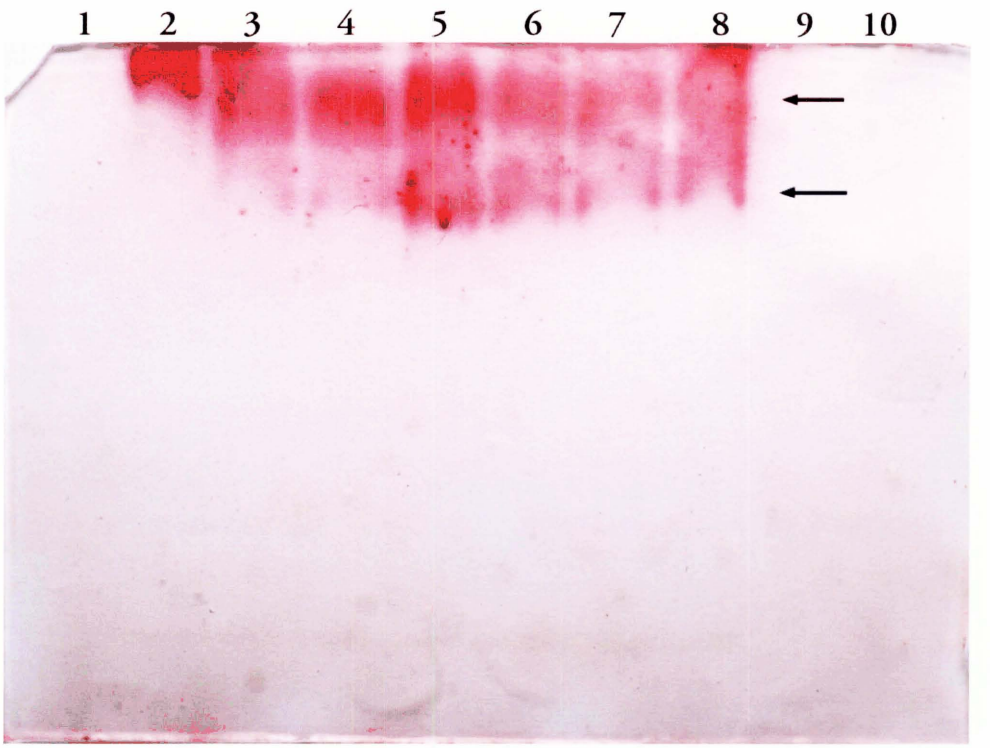
Lanes: 1 - dye marker (5μL).
2-9 - non Pb-treated shoot (65μL).

(B) Gel run with acetone-precipitated thawed frozen extracts of clone #7. Arrows indicate bands of enzyme activity after staining at 37 ° C for approx. 15 hours.

Lanes: 1 - dye marker (5μL).
2-4 - 500 μM Pb + 0.125 mM H-EDTA treated shoot (60μL).
5 - non Pb-treated shoot (60μL).
6-8 - 500 μM Pb + 0.125 mM H-EDTA treated shoot (60μL).



(A) Low pH acid phosphatase isozyme native gel, run with fresh concentrated shoot extracts from *Chamaecytisus palmensis* seedlings.



(B) Low pH acid phosphatase isozyme native gel, run with frozen concentrated shoot extracts from *Chamaecytisus palmensis* clone #7, after 7 days in 500 µM Pb plus 0.125 mM H-EDTA.

PLATE 44

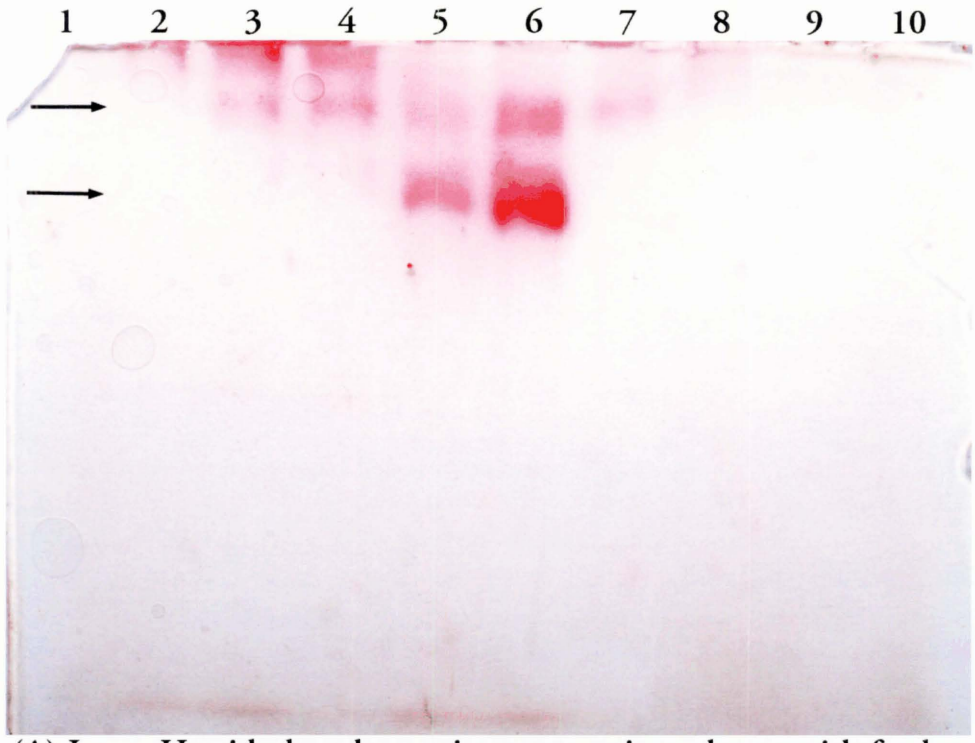
PLATE 45 Low pH native gels loaded with shoot extracts from *Chamaecytisus palmensis* clones #7 and #2 for acid phosphatase isozyme detection.

(A) Gel run with acetone-precipitated fresh and thawed frozen extracts of clone #7. Arrows indicate bands of enzyme activity after staining at 37 ° C for approx. 15 hours. (60 µL of extract loaded in each lane).

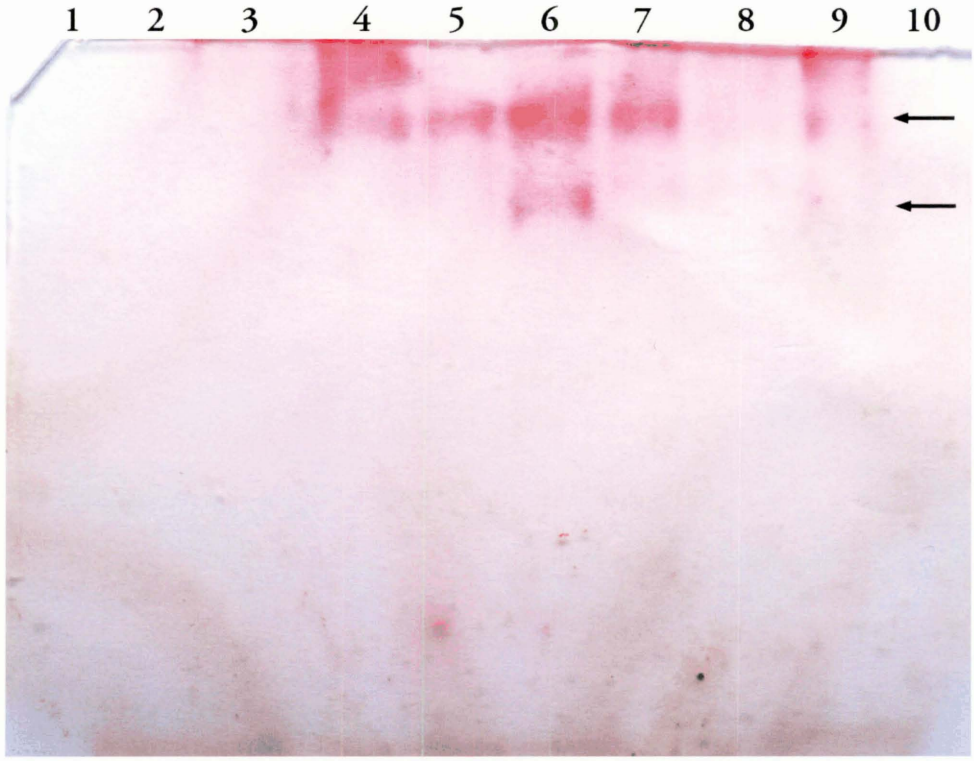
Lanes: 1 - dye marker (20µL).
2 & 3 - thawed frozen non Pb-treated shoot.
4 - thawed frozen 500 µM Pb-treated shoot.
5 - thawed frozen 500 µM Pb + 0.125 mM H-EDTA treated shoot.
6 - thawed frozen 500 µM Pb + 0.5 mM EDTA treated shoot.
7 - fresh non Pb-treated shoot.
8 - fresh 500 µM Pb-treated shoot.
9 - fresh 500 µM Pb + 0.125 mM H-EDTA treated shoot.
10 - fresh 500 µM Pb + 0.5 mM EDTA treated shoot.

(B) Gel run with acetone-precipitated fresh and thawed frozen extracts of clones #7 and #2. Arrows indicate bands of enzyme activity after staining at 37° C for approx. 15 hours. (60 µL in each lane).

Lanes: 3 - fresh non Pb-treated clone #2 shoot.
4 - fresh 500 µM Pb-treated clone #2 shoot.
5 - fresh 500 µM Pb + 0.5 mM EDTA treated clone #2 shoot.
6 - thawed frozen non Pb-treated clone #7 shoot.
7 - thawed frozen 500 µM Pb-treated clone #7 shoot.
8 - thawed frozen 500 µM Pb + 0.125 mM H-EDTA treated clone #7 shoot.
9 - thawed frozen 500 µM Pb + 0.5 mM EDTA treated clone #7 shoot.



(A) Low pH acid phosphatase isozyme native gel, run with fresh and frozen concentrated shoot extracts from *Chamaecytisus palmensis* clone #7, after 7 days in 500 µM Pb, with and without chelation.



(B) Low pH acid phosphatase isozyme native gel, run with fresh and frozen concentrated shoot extracts from *Chamaecytisus palmensis* clones #2 & #7, after 7 days in 500 µM Pb, with and without chelation.

PLATE 45

APPENDIX I

SCANNING ELECTRON MICROSCOPY

Electron probe x-ray microanalysis

In general, all samples examined in the scanning electron microscope (SEM, Leica S440) were first freeze-fractured in liquid N_2, then mounted on aluminium stubs with carbon conductive paint (Pro Sci Tech, Qld., Aust.). They were then usually carbon coated (Edwards model) for approx. 5 minutes.

Once the specimen chamber was fully evacuated, the electron probe x-ray microanalysis (EPXMA, Oxford Link Isis) software was used to generate a spectrum of elemental composition on the sample in question, by exposing it to X-rays which caused the emission of electrons characteristic of each element present. Spectra could be generated on the entire specimen, or only on selected regions, depending on the nature of the enquiry.

When the initial spectrum generation was complete, typically 120 seconds, other software functions, such as peak identification, could be used to determine which elements were present, and label those peaks that represented the elements of interest. After that, the spectrum could be printed out, together with the desired labels.

Vacuum filtrated *P. radiata* needles

(a) Round I: *P. radiata* needles were partially submerged in approx. 20 ml $Pb(NO_3)_2$ solution, and placed under partial vacuum in a vacuum evaporator for 1.5-2 hrs (until air bubbles stopped emanating from needles). Two fascicles each from two FRI clones, Z and V, were exposed initially to 302 μM Pb^{2+}, 604 μM Pb^{2+}, and dH_2O as a control (fascicles were cut whilst submerged in dH_2O to prevent damage to cells at the cut end). Afterwards, the needles remained in this solution, at atmospheric pressure, for approx. one month. Gross morphological

(Appendix I cont.)

changes were observed both with the naked eye and the light microscope. Needles vacuum filtrated at 604 μM Pb^{2+} were washed three times with dH_2O, then sent to the SEM for for Pb detection.

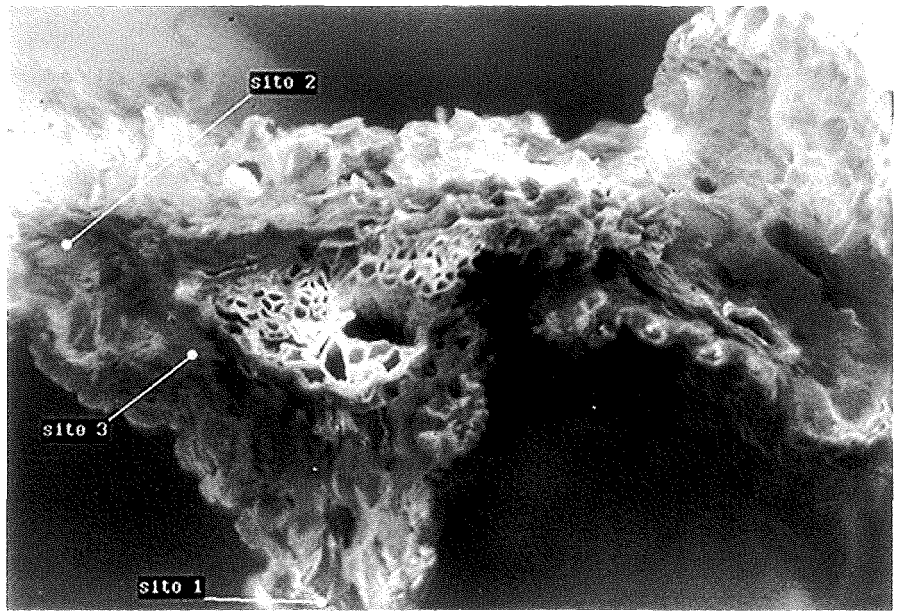
(b) Round II: Needles were vacuum filtrated at 1.208 mM Pb^{2+} until air bubbles were no longer seen, then left soaking in the solution for 4 days. Gross morphological changes were scored, the needles were washed three times with dH_2O, prepared for the SEM, and examined for Pb deposition.

After the needles from clones Z and V were removed from the desiccator they remained in the treatment solutions. After four days colour change was evident on the Pb-treated needles, particularly the 604 μM ones, including mottling, blotchiness and bands of discolouration. Under the light microscope the discolouration proved to be subcutaneous and quite dense in patches. In contrast, the controls looked quite healthy. At seven days the 302 μM needles were becoming blotchy and the 604 μM needles were becoming more discoloured. At 1 month, this effect was more obvious, the 302 μM needles being partially discoloured while the 604 μM needles were extensively discoloured. At this stage, the control retained most of its colour, displayed traces of mottling and little discolouration. There was no apparent difference between the responses of the 2 clones, Z and V.

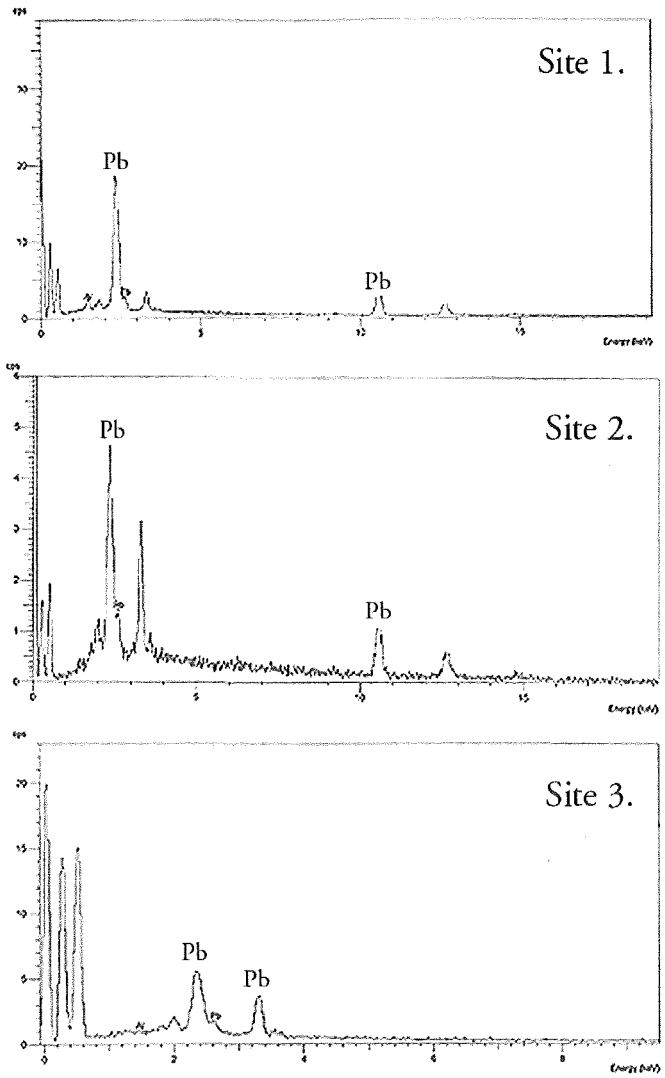
Sections of the 604 μM needles examined with EPXMA did not show measurable Pb deposition. As no reliable Pb peak could be detected with the SEM at this level it was decided that, if possible, a threshold response level should be found and the treatment concentration was doubled to 1.208 mM. When needles were vacuum filtrated at 1.208 mM Pb, along with dH_2O controls, after 4 days soaking in the treatment solution the treated needles appeared considerably more affected than the controls, particularly at the cut ends. Reddish-brown discolouration was evident on all cut ends of treated needles for approx. 1 mm. When transverse sections of these were examined with EPXMA, significant Pb deposition was detected. Three separate sites within one transverse section were selected and labelled, and individual peak traces were generated on each (Plate 46). The maximum Pb levels detected ranged from approx. 5 counts per second (cps) for site 2, to approx. 20 cps for site 1.

PLATE 46 (A) Scanning electron micrograph of transverse section of *Pinus radiata* needle vacuum filtrated in 400 mg L^{-1} Pb, illustrating three labeled sites chosen for Pb detection.

(B) Electron probe X-ray microanalysis (EPXMA) spectra generated from the three sites depicted in (A).



(A) SEM micrograph depicting three labeled sites in a transverse section of a *Pinus radiata* needle, vacuum filtrated at 400 mg/L Pb.



(B) EPXMA peak traces depicting magnitude of Pb deposition measured in counts per second at sites in (A).

PLATE 46

(Appendix I cont.)

Site 1 was closer to the epidermis of the needle than site 2. Site 3, at approx. 6 cps, was closest to the vascular bundle.

Excised *P. radiata* shoots in solution

(a) Round I: Ten shoots, each approx. 100 mm, were obtained from each of six different glasshouse grown clones designated as Q, R, S, V, X, and Z. Five shoots of each clone were placed in 10 ml $Pb(NO_3)_2$ solution at a concentration of 302 μM and five shoots were placed in 10 ml dH_2O as controls. Shoots were trimmed whilst submerged in dH_2O to prevent damage to the cells at the cut end.

The level of solution in each McCartney bottle was marked to enable topping up as necessary. All shoots were placed in a growth room with 16 h daylength at 22° C for 1 month. Observations were made of gross morphological changes such as tip burn, wilting, and colour change.

(b) Round II: Four Pb treatments and four dH_2O controls. The $Pb(NO_3)_2$ concentration was raised to 1.208 mM. Each glass vial contained 10 ml solution, placed in the growth room. Solutions were topped up as necessary. After 10 days, some fascicles were detached and sent to the SEM for analysis. Morphological observations were also carried out. After 28 days, two whole shoots (1 treatment, 1 control) were removed from solution, desiccated, and sent to the SEM for analysis.

In round I, after 8 days in solution, the needles on the Pb-treated shoots from clones Q, R, S, V, X, and Z began to appear slightly more mottled in comparison to the controls. The colour was no longer uniform, displaying lighter, chlorotic regions among the unaffected tissue. Some of the controls also displayed traces of mottling. By this stage, out of the 30 treated shoots, one or two had deteriorated badly and appeared to be dying but the majority were still viable.

After 20-24 days, the majority of the Pb-treated shoots were displaying needle tip-burn, compared to the controls, and the colour was fading. At the conclusion of the experiment, the shoots had not significantly deteriorated further and all Pb-treated shoots were in marginally poorer condition than the controls but

(Appendix I cont.)

it was not possible to determine if individual clones were affected by the treatment more than others.

When this experiment was repeated with higher Pb-concentration, (1.208 mM Pb) similar results were obtained. After 21 days external differences between treatments and controls were minimal and needle mottling was occurring in both sets, but not on every shoot. No trace of Pb could be found in needles sent for EPXMA after 10 days exposure to Pb but when whole shoots were examined after 28 days, definite Pb deposition was detected in the stem of the treated shoot whereas no Pb was detected in Pb-treated needles or any of the control material. Pb-treated stem sections were again analysed, examining regions both above and below the level of immersion to determine if differences between these regions could be detected, as prolonged contact with the Pb solution could possibly have obscured the results. Localised Pb deposition in the outer tissues (bark) of the immersed section was found compared with uniform homogeneous Pb deposition in the internal tissues of the non-immersed section.

APPENDIX J

FLAME A.A SPECTROMETER Pb SENSITIVITY TRIAL

Lead spiked *C. palmensis*

In a trial testing the Pb-sensitivity of the flame a.a. spectrometer, four *C. palmensis* seedling samples, (5g f.w.), were dry ashed at 550° C. Two samples had a Pb spike (2.0 ml of 100 ppm $Pb(NO_3)_2$) added to the crucibles prior to ashing and two acted as controls. Each ashed sample was dissolved in 10 ml of 2% HNO_3 (v/v) and was subsequently analysed by flame a.a.

Figure 32 depicts Pb levels measured by flame a.a spectrometry in *C. palmensis* seedling samples, including 0 Pb controls, and Pb-spiked specimens. The controls both indicate Pb levels of less than 0.5 ppm, and the spiked samples indicate Pb levels of approx. 11 ppm and 11.5 ppm respectively.

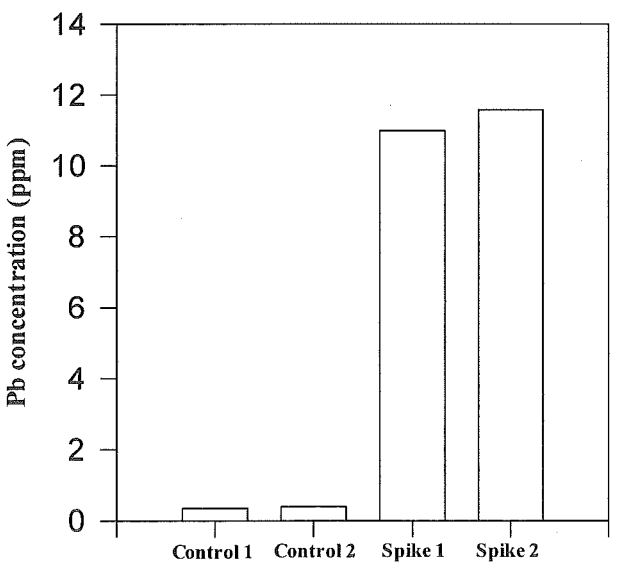


Figure 32. Pb-measurement sensitivity of flame a.a. on *Chamaecytisus palmensis*. 2 controls and 2 Pb-spiked with 2 ml of 100 ppm $Pb(NO_3)_2$. Values derived from mean absorbances interpolated against a calibration curve.

APPENDIX K

FLAME A.A. Pb CALIBRATION CURVE

Prior to flame a.a. analysis for Pb levels in each batch of experimental materials, the machine was calibrated with analytical grade 1000 ppm $Pb(NO_3)_2$ standard solution (BDH) diluted to 5, 10, 15, and 20 ppm with dH_2O. After zeroing the machine with 1 % HNO_3, 3 absorbance readings were taken at each level, at either 217 or 283 nm, and a calibration curve was constructed from the mean absorbance values. In table 24 a representative set of calibration data can be seen. In figure 33 the calibration curve derived from the data in table 24 is depicted along with the equation of the curve and its associated coefficient of variation.

Table 24. Pb Calibration data derived from flame a.a on Pb stock solutions at 283 nm. Three readings were taken at each level.

Pb concentration	Reading 1	Reading 2	Reading 3	Mean
5 ppm	0.103	0.097	0.109	0.103
10 ppm	0.203	0.196	0.210	0.203
15 ppm	0.306	0.307	0.304	0.306
20 ppm	0.384	0.398	0.395	0.392

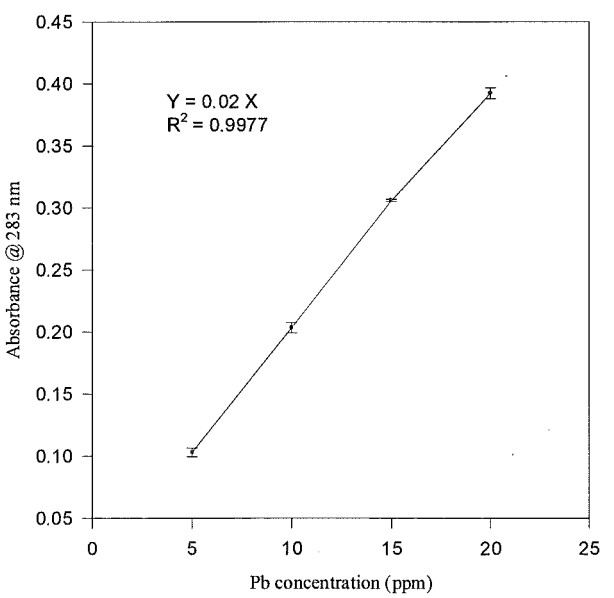


Figure 33. Flame a.a calibration curve generated from Pb stock solutions of 5, 10, 15, and 20 ppm, at 283 nm. Bars represent standard error (n=3).